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Method M
Monoclonal Purified

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CAUTION: US Federal law prohibits dispensing without prescription.

HOW SUPPLIED

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6 g Individual vial package NDC 52769-270-76
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HOW SUPPLIED

Immune Globulin Intravenous (Human), Polygam® S/D, is supplied in 2.5 g, 5 g or 10 g single use bottles. Each bottle of Immune Globulin Intravenous (Human), Polygam® S/D, is furnished with a suitable volume of Sterile Water for Injection, USP, a transfer device and an administration set which contains an integral airway and a 15 micron filter.
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EPOGEN® B
EPOETIN ALFA
RECOMBINANT
For Injection

DESCRIPTION

Erythropoietin is a glycoprotein which stimulates red blood cell production. It is produced in the kidney and stimulates the division and differentiation of committed erythroid progenitors in the bone marrow. EPOGEN® (Epoetin alfa), a 165 amino acid glycoprotein manufactured by recombinant DNA technology, has the same biological effects as endogenous erythropoietin.[1] It has a molecular weight of 30,400 daltons and is produced by mammalian cells into which the human erythropoietin gene has been introduced. The product contains the identical amino acid sequence of isolated natural erythropoietin.
EPOGEN® is formulated as a sterile, colorless liquid in an isotonic sodium chloride/sodium citrate buffered solution for intravenous (IV) or subcutaneous (SC) administration.
Single-dose, Preservative-free Vial: Each 1 mL of solution contains 2000, 3000, 4000 or 10,000 Units of Epoetin alfa, 2.5 mg Albumin (Human), 5.8 mg sodium citrate, 5.8 mg sodium chloride, and 0.06 mg citric acid in Water for Injection, USP (pH 6.9 ± 0.3). This formulation contains no preservative.
Multidose, Preserved Vial: 2 mL (20,000 Units, 10,000 Units/mL). Each 1 mL of solution contains 10,000 Units of Epoetin alfa, 2.5 mg Albumin (Human), 1.3 mg sodium citrate, 8.2 mg sodium chloride, 0.11 mg citric acid, and 1% benzyl alcohol as preservative in Water for Injection, USP (pH 6.1 ± 0.3).
Multidose, Preserved Vial: 1 mL (20,000 Units/mL). Each 1 mL of solution contains 20,000 Units of Epoetin alfa, 2.5 mg Albumin (Human), 1.3 mg sodium citrate, 8.2 mg sodium chloride, 0.11 mg citric acid, and 1% benzyl alcohol as preservative in Water for Injection, USP (pH 6.1 ± 0.3).

CLINICAL PHARMACOLOGY

Chronic Renal Failure Patients

Endogenous production of erythropoietin is normally regulated by the level of tissue oxygenation. Hypoxia and anemia generally increase the production of erythropoietin, which in turn stimulates erythropoiesis.[2] In normal subjects, plasma erythropoietin levels range from 0.01 to 0.03 Units/mL and increase up to 100- to 1000-fold during hypoxia or anemia.[2,3] In contrast, in patients with chronic renal failure (CRF), production of erythropoietin is impaired, and this erythropoietin deficiency is the primary cause of their anemia.[3,4]
Chronic renal failure is the clinical situation in which there is a progressive and usually irreversible decline in kidney function. Such patients may manifest the sequelae of renal dysfunction, including anemia, but do not necessarily require regular dialysis. Patients with end-stage renal disease (ESRD) are those patients with CRF who require regular dialysis or kidney transplantation for survival.
EPOGEN® has been shown to stimulate erythropoiesis in anemic patients with CRF, including both patients on dialysis and those who do not require regular dialysis.[4-13] The first evidence of a response to the three times weekly (TIW) administration of EPOGEN® is an increase in the reticulocyte count within 10 days, followed by increases in the red cell count, hemoglobin, and hematocrit, usually within 2 to 6 weeks.[4,5] Because of the length of time required for erythropoiesis—several days for erythroid progenitors to mature and be released into the circulation—a clinically significant increase in hematocrit is usually not observed in less than 2 weeks and may require up to 6 weeks in some patients. Once the hematocrit reaches the suggested target range (30% to 36%), that level can be sustained by EPOGEN® therapy in the absence of iron deficiency and concurrent illnesses.
The rate of hematocrit increase varies between patients and is dependent upon the dose of EPOGEN®, within a therapeutic range of approximately 50 to 300 Units/kg TIW.[4] A greater biologic response is not observed at doses exceeding 300 Units/kg TIW.[5] Other factors affecting the rate and extent of response include availability of iron stores, the baseline hematocrit, and the presence of concurrent medical problems.

Zidovudine-treated HIV-Infected Patients

Responsiveness to EPOGEN® in HIV-infected patients is dependent upon the endogenous serum erythropoietin level prior to treatment. Patients with endogenous serum erythropoietin levels ≤500 mUnits/mL, and who are receiving a dose of zidovudine ≤4200 mg/week, may respond to EPOGEN® therapy. Patients with endogenous serum erythropoietin levels >500 mUnits/mL do not appear to respond to EPOGEN® therapy. In a series of four clinical trials involving 255 patients, 60% to 80% of HIV-infected patients treated with zidovudine had endogenous serum erythropoietin levels ≤500 mUnits/mL.
Response to EPOGEN® in zidovudine-treated HIV-infected patients is manifested by reduced transfusion requirements and increased hematocrit.

Cancer Patients on Chemotherapy

Anemia in cancer patients may be related to the disease itself or the effect of concomitantly administered chemotherapeutic agents. EPOGEN® has been shown to increase hematocrit and decrease transfusion requirements after the first month of therapy (months 2 and 3), in anemic cancer patients undergoing chemotherapy.
A series of clinical trials enrolled 131 anemic cancer patients who were receiving cyclic cisplatin- or non cisplatin-containing chemotherapy. Endogenous baseline serum erythropoietin levels varied among patients in these trials with approximately 75% (n=83/110) having endogeneous serum erythropoietin levels ≤132 mUnits/mL, and approximately 4% (n=4/110) of patients having endogenous serum erythropoietin levels >500 mUnits/mL. In general, patients with lower baseline serum erythropoietin levels responded more vigorously to EPOGEN® than patients with higher baseline erythropoietin levels. Although no specific serum erythropoietin level can be stipulated above which patients would be unlikely to respond to EPOGEN® therapy, treatment of patients with grossly elevated serum erythropoietin levels (eg, >200 mUnits/mL) is not recommended.

Pharmacokinetics

Intravenously administered EPOGEN® is eliminated at a rate consistent with first order kinetics with a circulating half-life ranging from approximately 4 to 13 hours in patients with CRF. Within the therapeutic dose range, detectable levels of plasma erythropoietin are maintained for at least 24 hours.[7] After SC administration of EPOGEN® to patients with CRF, peak serum levels are achieved within 5 to 24 hours after administration and decline slowly thereafter. There is no apparent difference in half-life between patients not on dialysis whose serum creatinine levels were greater than 3, and patients maintained on dialysis.
In normal volunteers, the half-life of IV administered EPOGEN® is approximately 20% shorter than the half-life in CRF patients. The pharmacokinetics of EPOGEN® have not been studied in HIV-infected patients.

INDICATIONS AND USAGE

Treatment of Anemia of Chronic Renal Failure Patients

EPOGEN® is indicated for the treatment of anemia associated with CRF, including patients on dialysis (ESRD) and patients not on dialysis. EPOGEN® is indicated to elevate or maintain the red blood cell level (as manifested by the hematocrit or hemoglobin determinations) and to decrease the need for transfusions in these patients.
Non-dialysis patients with symptomatic anemia considered for therapy should have a hematocrit less than 30%.
EPOGEN® is not intended for patients who require immediate correction of severe anemia. EPOGEN® may obviate the need for maintenance transfusions but is not a substitute for emergency transfusion.
Prior to initiation of therapy, the patient's iron stores should be evaluated. Transferrin saturation should be at least 20% and ferritin at least 100 ng/mL. Blood pressure should be adequately controlled prior to initiation of EPOGEN® therapy, and must be closely monitored and controlled during therapy.
EPOGEN® should be administered under the guidance of a qualified physician (see DOSAGE AND ADMINISTRATION).

Treatment of Anemia in Zidovudine-treated HIV-Infected Patients

EPOGEN® is indicated for the treatment of anemia related to therapy with zidovudine in HIV-infected patients. EPOGEN® is indicated to elevate or maintain the red blood cell level (as manifested by the hematocrit or hemoglobin determinations) and to decrease the need for transfusions in these patients. EPOGEN® is not indicated for the treatment of anemia in HIV-infected patients due to other factors such as iron or folate deficiencies, hemolysis or gastrointestinal bleeding, which should be managed appropriately.
EPOGEN®, at a dose of 100 Units/kg TIW, is effective in decreasing the transfusion requirement and increasing the red blood cell level of anemic, HIV-infected patients treated with zidovudine, when the endogenous serum erythropoietin level is ≤500 mUnits/mL and when patients are receiving a dose of zidovudine ≤4200 mg/week.

Treatment of Anemia in Cancer Patients on Chemotherapy

EPOGEN® is indicated for the treatment of anemia in patients with non-myeloid malignancies where anemia is due to the effect of concomitantly administered chemotherapy. EPOGEN® is indicated to decrease the need for transfusions in patients who will be receiving concomitant chemotherapy for a minimum of 2 months. EPOGEN® is not indicated for the treatment of anemia in cancer patients due to other factors such as iron or folate deficiencies, hemolysis or gastrointestinal bleeding which should be managed appropriately.

Reduction of Allogeneic Blood Transfusion in Surgery Patients

EPOGEN® is indicated for the treatment of anemic patients (hemoglobin >10 to ≤ 13 g/dL) scheduled to undergo elective, noncardiac, nonvascular surgery to reduce the need for allogeneic blood transfusions.[14-16] EPOGEN® is indicated for patients at high risk for perioperative transfusions with significant, anticipated blood loss. EPOGEN® is not indicated for anemic patients who are willing to donate autologous blood. The safety of the perioperative use of EPOGEN® has been studied only in patients who are receiving anticoagulant prophylaxis.

CLINICAL EXPERIENCE: RESPONSE TO EPOGEN®

Chronic Renal Failure Patients

Response to EPOGEN® was consistent across all studies. In the presence of adequate iron stores (see IRON EVALUATION), the time to reach the target hematocrit is a function of the baseline hematocrit and the rate of hematocrit rise. The rate of increase in hematocrit is dependent upon the dose of EPOGEN® administered and individual patient

Continued on next page

Epogen—Cont.

variation. In clinical trials at starting doses of 50 to 150 Units/kg TIW, patients responded with an average rate of hematocrit rise of:

STARTING DOSE (TIW IV)	HEMATOCRIT INCREASE	
	POINTS/DAY	POINTS/2 WEEKS
50 Units/kg	0.11	1.5
100 Units/kg	0.18	2.5
150 Units/kg	0.25	3.5

Over this dose range, approximately 95% of all patients responded with a clinically significant increase in hematocrit, and by the end of approximately 2 months of therapy virtually all patients were transfusion-independent. Changes in the quality of life of patients treated with EPOGEN® were assessed as part of a Phase 3 clinical trial.[5,6] Once the target hematocrit (32% to 38%) was achieved, statistically significant improvements were demonstrated for most quality of life parameters measured, including energy and activity level, functional ability, sleep and eating behavior, health status, satisfaction with health, sex life, well-being, psychological effect, life satisfaction, and happiness. Patients also reported improvement in their disease symptoms. They showed a statistically significant increase in exercise capacity (VO_2 max), energy, and strength with a significant reduction in aching, dizziness, anxiety, shortness of breath, muscle weakness, and leg cramps.[8,17]

Patients on Dialysis

Thirteen clinical studies were conducted, involving IV administration to a total of 1010 anemic patients on dialysis for 986 patient-years of EPOGEN® therapy. In the three largest of these clinical trials, the median maintenance dose necessary to maintain the hematocrit between 30% to 36% was approximately 75 Units/kg TIW. In the US multicenter Phase 3 study, approximately 65% of the patients required doses of 100 Units/kg TIW, or less, to maintain their hematocrit at approximately 35%. Almost 10% of patients required a dose of 25 Units/kg, or less, and approximately 10% required a dose of more than 200 Units/kg TIW to maintain their hematocrit at this level.

A multicenter unit dose study was also conducted in 119 patients receiving peritoneal dialysis who self-administered EPOGEN® subcutaneously for approximately 109 patient-years of experience. Patients responded to EPOGEN® administered SC in a manner similar to patients receiving IV administration.[18]

Patients with CRF Not Requiring Dialysis

Four clinical trials were conducted in patients with CRF not on dialysis involving 181 patients treated with EPOGEN® for approximately 67 patient-years of experience. These patients responded to EPOGEN® therapy in a manner similar to that observed in patietns on dialysis. Patients with CRF not on dialysis demonstrated a dose-dependent and sustained increase in hematocrit when EPOGEN® was administered by either an IV or SC route, with similar rates of rise of hematocrit when EPOGEN® was administered by either route. Moreover, EPOGEN® doses of 75 to 150 Units/kg per week have been shown to maintain hematocrits of 36% to 38% for up to 6 months. Correcting the anemia of progressive renal failure will allow patients to remain active even though their renal function continues to decrease.[19–21]

Zidovudine-treated HIV-infected Patients

EPOGEN® has been studied in four placebo-controlled trials enrolling 297 anemic (hematocrit < 30%) HIV-infected (AIDS) patients receiving concomitant therapy with zidovudine (all patients were treated with Epoetin alfa manufactured by Amgen Inc.). In the subgroup of patients (69/125 EPOGEN® and 68/130 placebo) with prestudy endogenous serum erythropoietin levels ≤ 500 mUnits/mL, EPOGEN® reduced the mean cumulative number of units of blood transfused per patient by approximately 40% as compared to the placebo group.[22] Among those patients who required transfusions at baseline, 43% of patients treated with EPOGEN® versus 16% of placebo-treated patients were transfusion-independent during the second and third months of therapy. EPOGEN® therapy also resulted in significant increases in hematocrit in comparison to placebo. When examining the results according to the weekly dose of zidovudine received during month 3 of therapy, there was a statistically significant ($p < 0.003$) reduction in transfusion requirements in patients treated with EPOGEN® (n = 51) compared to placebo treated patients (n = 54) whose mean weekly zidovudine dose was ≤ 4200 mg/week.[22]

Approximately 17% of the patients with endogenous serum erythropoietin levels ≤ 500 mUnits/mL receiving EPOGEN® in doses from 100 to 200 Units/kg TIW achieved a hematocrit of 35% without administration of transfusions or significant reduction in zidovudine dose. In the subgroup of patients whose prestudy endogenous serum erythropoietin levels were > 500 mUnits/mL, EPOGEN® therapy did not reduce transfusion requirements or increase hematocrit, compared to the corresponding responses in placebo-treated patients. In a six month open-label EPOGEN® study, patients responded with decreased transfusion requirements and sustained increases in hematocrit and hemoglobin with doses of EPOGEN® up to 300 Units/kg TIW.[21–23]

Responsiveness to EPOGEN® therapy may be blunted by intercurrent infectious/inflammatory episodes and by an increase in zidovudine dosage. Consequently, the dose of EPOGEN® must be titrated based on these factors to maintain

Cancer Patients on Chemotherapy

EPOGEN® has been studied in a series of placebo-controlled, double-blind trials in a total of 131 anemic cancer patients. Within this group, 72 patients were treated with concomitant non cisplatin-containing chemotherapy regimens and 59 patients were treated with concomitant cisplatin-containing chemotherapy regimens. Patients were randomized to EPOGEN® 150 Units/kg or placebo subcutaneously TIW for 12 weeks.

EPOGEN® therapy was associated with a significantly ($p < 0.008$) greater hematocrit response than in the corresponding placebo-treated patients (see table).[22]

HEMATOCRIT (%): MEAN CHANGE FROM BASELINE TO FINAL VALUE*

STUDY	*EPOGEN®*	*PLACEBO*
Chemotherapy	7.6	1.3
Cisplatin	6.9	0.6

* Significantly higher in EPOGEN® patients than in placebo patients ($p<0.008$)

In the two types of chemotherapy studies (utilizing an EPOGEN® dose of 150 Units/kg TIW), the mean number of units of blood transfused per patient after the first month of therapy was significantly ($p < 0.02$) lower in patients treated with EPOGEN® (0.71 units in months 2, 3) than in corresponding placebo-treated patients (1.84 units in months 2, 3). Moreover, the proportion of patients transfused during months 2 and 3 of therapy combined was significantly ($p < 0.03$) lower in the patients treated with EPOGEN® than in the corresponding placebo-treated patients (22% vs 43%).[22]

Comparable intensity of chemotherapy in the EPOGEN® and placebo groups in the chemotherapy trials was suggested by a similar area under the neutrophil time curve in patients treated with EPOGEN® and placebo-treated patients as well as by a similar proportion of patients in groups treated with EPOGEN® and placebo-treated groups whose absolute neutrophil counts fell below 1000 cells/μL. Available evidence suggests that patients with lymphoid and solid cancers respond equivalently to EPOGEN® therapy, and that patients with or without tumor infiltration of the bone marrow respond equivalently to EPOGEN® therapy.

Surgery Patients

EPOGEN® has been studied in a placebo-controlled, double-blind trial enrolling 316 patients scheduled for major, elective orthopedic hip or knee surgery who were expected to require ≥ 2 units of blood and who were not able or willing to participate in an autologous blood donation program. Based on previous studies which demonstrated that pretreatment hemoglobin is a predictor of risk of receiving transfusion,[16,24] patients were stratified into one of three groups based on their pretreatment hemoglobin [≤ 10 (n = 2), > 10 to ≤ 13 (n = 96), and > 13 to ≤ 15 g/dL (n = 218)] and then randomly assigned to receive 300 Units/kg EPOGEN®, 100 Units/kg EPOGEN® or placebo by SC injection for 10 days before surgery, on the day of surgery, and for four days after surgery.[14] All patients received oral iron and a low-dose post-operative warfarin regimen.[14]

Treatment with EPOGEN® 300 Units/kg significantly ($p = 0.024$) reduced the risk of allogeneic transfusion in patients with a pretreatment hemoglobin of > 10 to ≤ 13 g/dL; 5/31 (16%) of EPOGEN® 300 Units/kg, 6/26 (23%) of EPOGEN® 100 Units/kg, and 13/29 (45%) of placebo-treated patients were transfused.[14] There was no significant difference in the number of patients transfused between EPOGEN® (9% 300 Units/kg, 6% 100 Units/kg) and placebo (13%) in the > 13 to ≤ 15 g/dL hemoglobin stratum. There were too few patients in the ≤ 10 g/dL group to determine if EPOGEN® is useful in this hemoglobin strata. In the > 10 to ≤ 13 g/dL pretreatment stratum, the mean number of units transfused per EPOGEN® treated patient (0.45 units blood for 300 Units/kg, 0.42 units blood for 100 Units/kg) was less than the mean transfused per placebo-treated patient (1.14 units) (overall $p = 0.028$). In addition, mean hemoglobin, hematocrit and reticulocyte counts increased significantly during the presurgery period in patients treated with EPOGEN®.[14]

EPOGEN® was also studied in an open-label, parallel-group trial enrolling 145 subjects with a pretreatment hemoglobin level of ≥ 10 to ≤ 13 g/dL who were scheduled for major orthopedic hip or knee surgery and who were not participating in an autologous program.[15] Subjects were randomly assigned to receive one of two SC dosing regimens of EPOGEN® (600 Units/kg once weekly for three weeks prior to surgery and on the day of surgery, or 300 Units/kg once daily for 10 days prior to surgery, on the day of surgery and for 4 days after surgery). All subjects received oral iron and appropriate pharmacologic anticoagulation therapy.

From pretreatment to presurgery, the mean increase in hemoglobin in the 600 Units/kg weekly group (1.44 g/dL) was greater than observed in the 300 Units/kg daily group.[15] The mean increase in absolute reticulocyte count was smaller in the weekly group ($0.11 \times 10^6/mm^3$) compared to the daily group ($0.17 \times 10^6/mm^3$). Mean hemoglobin levels were similar for the two treatment groups throughout the postsurgical period.

The erythropoietic response observed in both treatment groups resulted in similar transfusion rates [11/69 (16%) in Units/kg daily group].[15] The mean number of units transfused per subject was approximately 0.3 units in both treatment groups.

CONTRAINDICATIONS

EPOGEN® is contraindicated in patients with:

1. Uncontrolled hypertension.
2. Known hypersensitivity to mammalian cell-derived products.
3. Known hypersensitivity to Albumin (Human).

WARNINGS

Pediatric Use

The multidose preserved formulation contains benzyl alcohol. Benzyl alcohol has been reported to be associated with an increased incidence of neurological and other complications in premature infants which are sometimes fatal. The safety and effectiveness of Epoetin alfa in pediatric patients have not been established.

Thrombotic Events and Increased Mortality

A randomized, prospective trial of 1265 hemodialysis patients with clinically evident cardiac disease (ischemic heart disease or congestive heart failure) was conducted in which patients were assigned to EPOGEN® treatment targeted to a maintenance hematocrit of either 42 ± 3% or 30 ± 3%. Increased mortality was observed in 634 patients randomized to a target hematocrit of 42% [221 deaths (35% mortality)] compared to 631 patients targeted to remain at a hematocrit of 30% [185 deaths (29% mortality)]. The reason for the increased mortality observed in these studies is not known, however the incidence of non-fatal myocardial infarctions (3.1% vs 2.3%), vascular access thromboses (39% vs 29%), and all other thrombotic events (22% vs 18%) were also higher in the group randomized to achieve a hematocrit of 42%.

Increased mortality was also observed in a randomized placebo-controlled study of EPOGEN® in patients who did not have CRF who were undergoing coronary artery bypass surgery (7 deaths in 126 patients randomized to EPOGEN® versus no deaths among 56 patients receiving placebo). Four of these deaths occurred during the period of study drug administration and all 4 deaths were associated with thrombotic events. While the extent of the population affected is unknown, in patients at risk for thrombosis, the anticipated benefits of EPOGEN® treatment should be weighed against the potential for increased risks associated with therapy.

Chronic Renal Failure Patients

Hypertension: Patients with uncontrolled hypertension should not be treated with EPOGEN®; blood pressure should be controlled adequately before initiation of therapy. Up to 80% of patients with CRF have a history of hypertension.[25] Although there does not appear to be any direct pressor effects of EPOGEN®, blood pressure may rise during EPOGEN® therapy. During the early phase of treatment when the hematocrit is increasing, approximately 25% of patients on dialysis may require initiation of, or increase in, antihypertensive therapy. Hypertensive encephalopathy and seizures have been observed in patients with CRF treated with EPOGEN®.

Special care should be taken to closely monitor and aggressively control blood pressure in patients treated with EPOGEN®. Patients should be advised as to the importance of compliance with antihypertensive therapy and dietary restrictions. If blood pressure is difficult to control by initiation of appropriate measures, the hematocrit may be reduced by decreasing or withholding the dose of EPOGEN®. A clinically significant decrease in hematocrit may not be observed for several weeks.

It is recommended that the dose of EPOGEN® be decreased if the hematocrit increase exceeds 4 points in any 2-week period, because of the possible association of excessive rise of hematocrit with an exacerbation of hypertension. In CRF patients on hemodialysis with clinically evident ischemic heart disease or congestive heart failure, the hematocrit should be managed carefully, not to exceed 36% (SEE THROMBOTIC EVENTS).

Seizures: Seizures have occurred in patients with CRF participating in EPOGEN® clinical trials.

In patients on dialysis, there was a higher incidence of seizures during the first 90 days of therapy (occurring in approximately 2.5% of patients) as compared with later time points.

Given the potential for an increased risk of seizures during the first 90 days of therapy, blood pressure and the presence of premonitory neurologic symptoms should be monitored closely. Patients should be cautioned to avoid potentially hazardous activities such as driving or operating heavy machinery during this period.

While the relationship between seizures and the rate of rise of hematocrit is uncertain, it is recommended that the dose of EPOGEN® be decreased if the hematocrit increase exceeds 4 points in any 2-week period.

Thrombotic Events: During hemodialysis, patients treated with EPOGEN® may require increased anticoagulation with heparin to prevent clotting of the artificial kidney (see ADVERSE REACTIONS for more information about thrombotic events).

Other thrombotic events (eg, myocardial infarction, cerebrovascular accident, transient ischemic attack) have occurred in clinical trials at an annualized rate of less than 0.5 events per patient per year of EPOGEN® therapy. These trials were conducted in patients with CRF (whether on dialysis or not) in whom the target hematocrit was 32% to 40%. However, the risk of thrombotic events, including

tients with ischemic heart disease or congestive heart failure receiving EPOGEN® therapy with the goal of reaching a normal hematocrit (42%) as compared to a target hematocrit of 30%. Patients with pre-existing cardiovascular disease should be monitored closely.

Zidovudine-treated HIV-infected Patients

In contrast to CRF patients, EPOGEN® therapy has not been linked to exacerbation of hypertension, seizures, and thrombotic events in HIV-infected patients.

PRECAUTIONS

The parenteral administration of any biologic product should be attended by appropriate precautions in case allergic or other untoward reactions occur (see CONTRAINDICATIONS). In clinical trials, while transient rashes were occasionally observed concurrently with EPOGEN® therapy, no serious allergic or anaphylactic reactions were reported (see ADVERSE REACTIONS for more information regarding allergic reactions).

The safety and efficacy of EPOGEN® therapy have not been established in patients with a known history of a seizure disorder or underlying hematologic disease (eg, sickle cell anemia, myelodysplastic syndromes, or hypercoagulable disorders).

In some female patients, menses have resumed following EPOGEN® therapy; the possibility of pregnancy should be discussed and the need for contraception evaluated.

Hematology

Exacerbation of porphyria has been observed rarely in patients with CRF treated with EPOGEN®. However, EPOGEN® has not caused increased urinary excretion of porphyrin metabolites in normal volunteers, even in the presence of a rapid erythropoietic response. Nevertheless, EPOGEN® should be used with caution in patients with known porphyria.

In preclinical studies in dogs and rats, but not in monkeys, EPOGEN® therapy was associated with subclinical bone marrow fibrosis. Bone marrow fibrosis is a known complication of CRF in humans and may be related to secondary hyperparathyroidism or unknown factors. The incidence of bone marrow fibrosis was not increased in a study of patients on dialysis who were treated with EPOGEN® for 12 to 19 months, compared to the incidence of bone marrow fibrosis in a matched group of patients who had not been treated with EPOGEN®.

Hematocrit in CRF patients should be measured twice a week, zidovudine-treated HIV-infected and cancer patients should have hematocrit measured once a week until hematocrit has been stabilized, and measured periodically thereafter.

Delayed or Diminished Response

If the patient fails to respond or to maintain a response to doses within the recommended dosing range, the following etiologies should be considered and evaluated:

1. Iron deficiency: Virtually all patients will eventually require supplemental iron therapy (see IRON EVALUATION).
2. Underlying infectious, inflammatory, or malignant processes.
3. Occult blood loss.
4. Underlying hematologic diseases (ie, thalassemia, refractory anemia, or other myelodysplastic disorders).
5. Vitamin deficiencies: Folic acid or vitamin B12.
6. Hemolysis.
7. Aluminum intoxication.
8. Osteitis fibrosa cystica.

Iron Evaluation

During EPOGEN® therapy, absolute or functional iron deficiency may develop. Functional iron deficiency, with normal ferritin levels but low transferrin saturation, is presumably due to the inability to mobilize iron stores rapidly enough to support increased erythropoiesis. Transferrin saturation should be at least 20% and ferritin should be at least 100 ng/mL.

Prior to and during EPOGEN® therapy, the patient's iron status, including transferrin saturation (serum iron divided by iron binding capacity) and serium ferritin, should be evaluated. Virtually all patients will eventually require supplemental iron to increase or maintain transferrin saturation to levels which will adequately support erythropoiesis stimulated by EPOGEN®. All surgery patients being treated with EPOGEN® should receive adequate iron supplementation throughout the course of therapy in order to support erythropoiesis and avoid depletion of iron stores.

Drug Interaction

No evidence of interaction of EPOGEN® with other drugs was observed in the course of clinical trials.

Carcinogenesis, Mutagenesis, and Impairment of Fertility

Carcinogenic potential of EPOGEN® has not been evaluated. EPOGEN® does not induce bacterial gene mutation (Ames Test), chromosomal aberrations in mammalian cells, micronuclei in mice, or gene mutation at the HGPRT locus. In female rats treated IV with EPOGEN®, there was a trend for slightly increased fetal wastage at doses of 100 and 500 Units/kg.

Pregnancy Category C

EPOGEN® has been shown to have adverse effects in rats when given in doses 6 times the human dose. There are no adequate and well-controlled studies in pregnant women. EPOGEN® should be used during pregnancy only if potential benefit justifies the potential risk to the fetus.

In studies of female rats, there were decreases in body weight gain, delays in appearance of abdominal hair, delayed eyelid opening, delayed ossification, and decreases in the number of caudal vertebrae in the F1 fetuses of the 500 Units/kg group. In female rats treated IV, there was a trend for slightly increased fetal wastage at dosages of 100 and 500 Units/kg. EPOGEN® has not shown any adverse effect at doses as high as 500 Units/kg in pregnant rabbits (from day 6 to 18 of gestation).

Nursing Mothers

Postnatal observations of the live offspring (F1 generation) of female rats treated with EPOGEN® during gestation and lactation revealed no effect of EPOGEN® at doses of up to 500 Units/kg. There were, however, decreases in body weight gain, delays in appearance of abdominal hair, eyelid opening, and decreases in the number of caudal vertebrae in the F1 fetuses of the 500 Units/kg group. There were no EPOGEN®-related effects on the F2 generation fetuses.

It is not known whether EPOGEN® is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when EPOGEN® is administered to a nursing woman.

Pediatric Use

The safety and effectiveness of EPOGEN® in pediatric patients have not been established (see WARNINGS).

Chronic Renal Failure Patients

Patients with CRF Not Requiring Dialysis

Blood pressure and hematocrit should be monitored no less frequently than for patients maintained on dialysis. Renal function and fluid and electrolyte balance should be closely monitored, as an improved sense of well-being may obscure the need to initiate dialysis in some patients.

Hematology: Sufficient time should be allowed to determine a patient's responsiveness to a dosage of EPOGEN® before adjusting the dose. Because of the time required for erythropoiesis and the red cell half-life, an interval of 2 to 6 weeks may occur between the time of a dose adjustment (initiation, increase, decrease, or discontinuation) and a significant change in hematocrit.

In order to avoid reaching the suggested target hematocrit too rapidly, or exceeding the suggested target range (hematocrit of 30% to 36%), the guidelines for dose and frequency of dose adjustments (see DOSAGE AND ADMINISTRATION) should be followed.

For patients who respond to EPOGEN® with a rapid increase in hematocrit (eg, more than 4 points in any 2-week period), the dose of EPOGEN® should be reduced because of the possible association of excessive rate of rise of hematocrit with an exacerbation of hypertension.

The elevated bleeding time characteristic of CRF decreases toward normal after correction of anemia in patients treated with EPOGEN®. Reduction of bleeding time also occurs after correction of anemia by transfusion.

Laboratory Monitoring: The hematocrit should be determined twice a week until it has stabilized in the suggested target range and the maintenance dose has been established. After any dose adjustment, the hematocrit should also be determined twice weekly for at least 2 to 6 weeks until it has been determined that the hematocrit has stabilized in response to the dose change. The hematocrit should then be monitored at regular intervals.

A complete blood count with differential and platelet count should be performed regularly. During clinical trials, modest increases were seen in platelets and white blood cell counts. While these changes were statistically significant, they were not clinically significant and the values remained within normal ranges.

In patients with CRF, serum chemistry values [including blood urea nitrogen (BUN), uric acid, creatinine, phosphorus, and potassium] should be monitored regularly. During clinical trials in patients on dialysis, modest increases were seen in BUN, creatinine, phosphorus, and potassium. In some patients with CRF not on dialysis, treated with EPOGEN®, modest increases in serum uric acid and phosphorus were observed. While changes were statistically significant, the values remained within the ranges normally seen in patients with CRF.

Diet: As the hematocrit increases and patients experience an improved sense of well-being and quality of life, the importance of compliance with dietary and dialysis prescriptions should be reinforced. In particular, hyperkalemia is not uncommon in patients with CRF. In US studies in patients on dialysis, hyperkalemia has occurred at an annualized rate of approximately 0.11 episodes per patient-year of EPOGEN® therapy, often in association with poor compliance to medication, diet, and/or dialysis.

Dialysis Management: Therapy with EPOGEN® results in an increase in hematocrit and a decrease in plasma volume which could affect dialysis efficiency. In studies to date, the resulting increase in hematocrit did not appear to adversely affect dialyzer function[9,10] or the efficiency of high flux hemodialysis.[11] During hemodialysis, patients treated with EPOGEN® may require increased anticoagulation with heparin to prevent clotting of the artificial kidney.

Patients who are marginally dialyzed may require adjustments in their dialysis prescription. As with all patients on dialysis, the serum chemistry values (including BUN, creatinine, phosphorus, and potassium) in patients treated with EPOGEN® should be monitored regularly to assure the adequacy of the dialysis prescription.

Information for Patients: In those situations in which the physician determines that a home dialysis patient can safely and effectively self-administer EPOGEN®, the patient should be instructed as to the proper dosage and administration. Home dialysis patients should be referred to the full "Information for Home Dialysis Patients" insert; it is not a disclosure of all possible effects. Patients should be informed of the signs and symptoms of allergic drug reaction and advised of appropriate actions. If home use is prescribed for a home dialysis patient, the patient should be thoroughly instructed in the importance of proper dispos- and cautioned against the reuse of needles, syringes, drug product. A puncture-resistant container for the di- posal of used syringes and needles should be available to th- patient. The full container should be disposed of, accordin- to the directions provided by the physician.

Renal Function: In patients with CRF not on dialysis, re- nal function and fluid and electrolyte balance should be closely monitored, as an improved sense of well-being may obscure the need to initiate dialysis in some patients. In pa- tients with CRF not on dialysis, placebo-controlled studies of progression of renal dysfunction over periods of greater than one year have not been completed. In shorter term tri- als in patients with CRF not on dialysis, changes in serum nine and creatinine clearance were not significantly differ- ent in patients treated with EPOGEN®, compared with pla- cebo-treated patients. Analysis of the slope of 1/serum creatinine versus time plots in these patients indicates no significant change in the slope after the initiation of EPO- GEN® therapy.

Zidovudine-treated HIV-infected Patients

Hypertension: Exacerbation of hypertension has not been observed in zidovudine-treated HIV-infected patients treated with EPOGEN®. However, EPOGEN® should be withheld in these patients if pre-existing hypertension is uncontrolled, and should not be started until blood pressure is controlled. In double-blind studies, a single seizure has been experienced by a patient treated with EPOGEN®.

Cancer Patients on Chemotherapy

Hypertension: Hypertension, associated with a significant increase in hematocrit, has been noted rarely in patients treated with EPOGEN®. Nevertheless, blood pressure in patients treated with EPOGEN® should be monitored carefully, particularly in patients with an underlying history of hypertension or cardiovascular disease.

Seizures: In double-blind, placebo-controlled trials, 3.2% (n=2/63) of patients treated with EPOGEN® and 2.9% (n=2/68) of placebo-treated patients had seizures. Seizures in 1.6% (n=1/63) of patients treated with EPOGEN® occurred in the context of a significant increase in blood pressure and hematocrit from baseline values. However, both patients treated with EPOGEN® also had underlying CNS pathology which may have been related to seizure activity.

Thrombotic Events: In double-blind, placebo-controlled trials, 3.2% (n=2/63) of patients treated with EPOGEN® and 11.8% (n=8/68) of placebo-treated patients had thrombotic events (eg, pulmonary embolism, cerebrovascular accident).

Growth Factor Potential: EPOGEN® is a growth factor that primarily stimulates red cell production. However, the possibility that EPOGEN® can act as a growth factor for any tumor type, particularly myeloid malignancies, cannot be excluded.

Surgery patients

Thrombotic/Vascular Events: In perioperative clinical trials with orthopedic patients, the overall incidence of thrombotic/vascular events was similar in Epoetin alfa and placebo-treated patients who had a pretreatment hemoglobin of >10 to ≤13 g/dL. In patients with a hemoglobin of >13 g/dL treated with 300 Units/kg of Epoetin alfa, the possibility that EPOGEN® treatment may be associated with an increased risk of postoperative thrombotic/vascular events cannot be excluded.[14-16]

In one study in which Epoetin alfa was administered in the perioperative period to patients undergoing coronary artery bypass graft surgery, there were seven deaths in the group treated with Epoetin alfa (n=126) and no deaths in the placebo-treated group (n=56). Among the seven deaths in the patients treated with Epoetin alfa, four were at the time of therapy (between study day 2 and 8). The four deaths at the time of therapy (3%) were associated with thrombotic/vascular events. A causative role of Epoetin alfa cannot be excluded (see WARNINGS).

Hypertension: Blood pressure may rise in the perioperative period in patients being treated with EPOGEN®. Therefore, blood pressure should be monitored carefully.

ADVERSE REACTIONS

Chronic Renal Failure Patients

EPOGEN® is generally well-tolerated. The adverse events reported are frequent sequelae of CRF and are not necessarily attributable to EPOGEN® therapy. In double-blind, placebo-controlled studies involving over 300 patients with CRF, the events reported in greater than 5% of patients treated with EPOGEN® during the blinded phase were:

PERCENT OF PATIENTS REPORTING EVENT

Event	Patients Treated with EPOGEN® (n = 200)	Placebo-Treated Patients (n =135)
Hypertension	24%	19%
Headache	16%	12%
Arthralgias	11%	6%
Nausea	11%	9%
Edema	9%	10%
Fatigue	9%	14%
Diarrhea	9%	6%
Vomiting	8%	5%
Chest Pain	7%	9%
Skin Reaction, Administration Site	7%	12%

Continued on next page

Epogen—Cont.

Asthenia	7%	12%
Dizziness	7%	13%
Clotted Access	7%	2%

Significant adverse events of concern in patients with CRF treated in double-blind, placebo-controlled trials occurred in the following percent of patients during the blinded phase of the studies:

Seizure	1.1%	1.1%
CVA/TIA	0.4%	0.6%
MI	0.4%	1.1%
Death	0%	1.7%

In the US EPOGEN® studies in patients on dialysis (over 567 patients), the incidence (number of events per patient-year) of the most frequently reported adverse events were hypertension (0.75), headache (0.40), tachycardia (0.31), nausea/vomiting (0.26), clotted vascular access (0.25), shortness of breath (0.14), hyperkalemia (0.11), and diarrhea (0.11). Other reported events occurred at a rate of less than 0.10 events per patient per year.

Events reported to have occurred within several hours of administration of EPOGEN® were rare, mild, and transient, and included injection site stinging in dialysis patients and flu-like symptoms such as arthralgias and myalgias.

In all studies analyzed to date, EPOGEN® administration was generally well-tolerated, irrespective of the route of administration.

Hypertension: Increases in blood pressure have been reported in clinical trials, often during the first 90 days of therapy. On occasion, hypertensive encephalopathy and seizures have been observed in patients with CRF treated with EPOGEN®. When data from all patients in the US Phase 3 multicenter trial were analyzed, there was an apparent trend of more reports of hypertensive adverse events in patients on dialysis with a faster rate of rise of hematocrit (greater than 4 hematocrit points in any 2-week period). However, in a double-blind, placebo-controlled trial, hypertensive adverse events were not reported at an increased rate in the group treated with EPOGEN® (150 Units/kg TIW) relative to the placebo group.

Seizures: There have been 47 seizures in 1010 patients on dialysis treated with EPOGEN® in clinical trials, with an exposure of 986 patient-years for a rate of approximately 0.048 events per patient-year. However, there appeared to be a higher rate of seizures during the first 90 days of therapy (occurring in approximately 2.5% of patients) when compared to subsequent 90-day periods. The baseline incidence of seizures in the untreated dialysis population is difficult to determine; it appears to be in the range of 5% to 10% per patient-year.[26-28]

Thrombotic Events: In clinical trials where the maintenance hematocrit was 35 ± 3% on EPOGEN®, clotting of the vascular access (A-V shunt) has occurred at an annualized rate of about 0.25 events per patient-year, and other thrombotic events (eg, myocardial infarction, cerebral vascular accident, transient ischemic attack, and pulmonary embolism) occurred at a rate of 0.04 events per patient-year. In a separate study of 1111 untreated dialysis patients, clotting of the vascular access occurred at a rate of 0.50 events per patient-year. However, in CRF patients on hemodialysis who also had clinically evident ischemic heart disease or congestive heart failure, the risk of A-V shunt thrombosis was higher (39% vs 29%, $p < 0.001$), and myocardial infarctions, vascular ischemic events, and venous thrombosis were increased, in patients targeted to a hematocrit of 42 ± 3% compared to those maintained at 30 ± 3% (see WARNINGS).

In patients treated with commercial EPOGEN®, there have been rare reports of serious or unusual thrombo-embolic events including migratory thrombophlebitis, microvascular thrombosis, pulmonary embolus, and thrombosis of the retinal artery, and temporal and renal veins. A causal relationship has not been established.

Allergic Reactions: There have been no reports of serious allergic reactions or anaphylaxis associated with EPOGEN® administration during clinical trials. Skin rashes and urticaria have been observed rarely and when reported have generally been mild and transient in nature.

There have been rare reports of potentially serious allergic reactions including urticaria with associated respiratory symptoms or circumoral edema, or urticaria alone. Most reactions occurred in situations where a causal relationship could not be established. Symptoms recurred with rechallenge in a few instances, suggesting that allergic reactivity may occasionally be associated with EPOGEN® therapy.

There has been no evidence for development of antibodies to erythropoietin in patients tested to date, including those receiving EPOGEN® for over 4 years. Nevertheless, if an anaphylactoid reaction occurs, EPOGEN® should be immediately discontinued and appropriate therapy initiated.

Zidovudine-treated HIV-infected Patients

Adverse events reported in clinical trials with EPOGEN® in zidovudine-treated HIV-infected patients were consistent with the progression of HIV infection. In double-blind, placebo-controlled studies of three-months duration involving approximately 300 zidovudine-treated HIV-infected patients, adverse events with an incidence of ≥ 10% in either patients treated with EPOGEN® or placebo-treated patients were:

PERCENT OF PATIENTS REPORTING EVENT

Event	Patients Treated with EPOGEN® (n = 144)	Placebo-Treated Patients (n = 153)
Pyrexia	38%	29%
Fatigue	25%	31%
Headache	19%	14%
Cough	18%	14%
Diarrhea	16%	18%
Rash	16%	8%
Congestion, Respiratory	15%	10%
Nausea	15%	12%
Shortness of Breath	14%	13%
Asthenia	11%	14%
Skin Reaction, Medication Site	10%	7%
Dizziness	9%	10%

There were no statistically significant differences between treatment groups in the incidence of the above events.

In the 297 patients studied, EPOGEN® was not associated with significant increases in opportunistic infections or mortality.[22] In 71 patients from this group treated with EPOGEN® at 150 Units/kg TIW, serum p24 antigen levels did not appear to increase.[23] Preliminary data showed no enhancement of HIV replication in infected cell lines in vitro.[22] Peripheral white blood cell and platelet counts are unchanged following EPOGEN® therapy.

Allergic Reactions: Two zidovudine-treated HIV-infected patients had urticarial reactions within 48 hours of their first exposure to study medication. One patient was treated with EPOGEN® and one was treated with placebo (EPOGEN® vehicle alone). Both patients had positive immediate skin tests against their study medication with a negative saline control. The basis for this apparent pre-existing hypersensitivity to components of the EPOGEN® formulation is unknown but may be related to HIV-induced immunosuppression or prior exposure to blood products.

Seizures: In double-blind and open-label trials of EPOGEN® in zidovudine-treated HIV-infected patients, 10 patients have experienced seizures.[22] In general, these seizures appear to be related to underlying pathology, such as meningitis or cerebral neoplasms, not EPOGEN® therapy.

Cancer Patients on Chemotherapy

Adverse experiences reported in clinical trials with EPOGEN® in cancer patients were consistent with the underlying disease state. In double-blind, placebo-controlled studies of up to 3 months duration involving 131 cancer patients, adverse effects with an incidence > 10% in either patients treated with EPOGEN® or placebo-treated patients were as indicated below:

PERCENT OF PATIENTS REPORTING EVENT

Event	Patients Treated with EPOGEN® (n = 63)	Placebo-Treated Patients (n = 68)
Pyrexia	29%	19%
Diarrhea	21%[a]	7%
Nausea	17%[b]	32%
Vomiting	17%	15%
Edema	17%[c]	1%
Asthenia	13%	16%
Fatigue	13%	15%
Shortness of Breath	13%	9%
Paresthesia	11%	6%
Upper Respiratory Infection	11%	4%
Dizziness	5%	12%
Trunk Pain	3%[d]	16%

[a] p = 0.03.
[b] p = 0.05.
[c] p = 0.01.
[d] p = 0.02.

Although some statistically significant differences between patients being treated with EPOGEN® and placebo-treated patients were noted, the overall safety profile of EPOGEN® appeared to be consistent with the disease process of advanced cancer. During double-blind and subsequent open-label therapy in which patients (n = 72 for total exposure to EPOGEN®) were treated for up to 32 weeks with doses as high as 927 Units/kg, the adverse experience profile of EPOGEN® was consistent with the progression of advanced cancer.

Based on comparable survival data and on the percentage of patients treated with EPOGEN® and placebo-treated patients who discontinued therapy due to death, disease progression, or adverse experiences (22% and 13%, respectively, $p = 0.25$), the clinical outcome in patients treated with EPOGEN® and placebo-treated patients appeared to be similar. Available data from animal tumor models and measurement of proliferation of solid tumor cells from clinical biopsy specimens in response to EPOGEN® suggest that EPOGEN® does not potentiate tumor growth. Nevertheless, as a growth factor, the possibility that EPOGEN® may potentiate growth of some tumors, particularly myeloid tumors, cannot be excluded. A randomized controlled Phase 3 study is currently ongoing to further evaluate this issue. The mean peripheral white blood cell count was unchanged following EPOGEN® therapy compared to the corresponding value in the placebo-treated group.

Surgery Patients

Adverse events with an incidence of ≥ 10% are shown in the following table:

PERCENT OF PATIENTS REPORTING EVENT

Event	Patients Treated with EPOGEN® 300 U/kg (n = 112)[a]	Patients Treated with EPOGEN® 100 U/kg (n = 101)[a]	Placebo-treated Patients (n = 103)[a]	Patients Treated with EPOGEN® 600 U/kg (n = 73)[b]	Patients Treated with EPOGEN® 300 Y/kg (n = 72)[b]
Pyrexia	51%	50%	60%	47%	42%
Nausea	48%	43%	45%	45%	56%
Constipation	43%	42%	43%	51%	53%
Skin reaction, Medication site	25%	19%	22%	26%	29%
Vomiting	22%	12%	14%	21%	29%
Skin Pain	18%	18%	17%	5%	4%
Pruritus	16%	16%	14%	14%	22%
Insomnia	13%	16%	13%	21%	18%
Headache	13%	11%	9%	10%	19%
Dizziness	12%	9%	12%	11%	21%
Urinary Tract Infection	12%	3%	11%	11%	8%
Hypertension	10%	11%	10%	5%	10%
Diarrhea	10%	7%	12%	10%	6%
Deep Venous Thrombosis	10%	3%	5%	0%[c]	0%[c]
Dyspepsia	9%	11%	6%	7%	8%
Anxiety	7%	2%	11%	11%	4%
Edema	6%	11%	8%	11%	7%

[a] Study including patients undergoing orthopedic surgery treated with EPOGEN® or placebo for 15 days
[b] Study including patients undergoing orthopedic surgery treated with EPOGEN® 600 Units/kg weekly × 4 or 300 Units/kg daily × 15
[c] Determined by clinical symptoms

Thrombotic/Vascular Events: In three double-blind, placebo-controlled orthopedic surgery studies, the rate of deep venous thrombosis (DVT) was similar among Epoetin alfa- and placebo-treated patients in the recommended population of patients with a pretreatment hemoglobin of > 10 to ≤ 13 g/dL.[14,16,24] However, in 2 of 3 orthopedic surgery studies, the overall rate (all pretreatment hemoglobin groups combined) of DVTs detected by postoperative ultrasonography and/or surveillance venography was higher in the group treated with Epoetin alfa than in the placebo-treated group (25% vs 9%). This finding was attributable to the difference in DVT rates observed in the subgroup of patients with pretreatment hemoglobin > 13 g/dL. However, the incidence of DVTs was within the range of that reported in the literature for orthopedic surgery patients.

In the orthopedic surgery study of patients with pretreatment hemoglobin of > 10 to ≤ 13 g/dL which compared dosing regimens (600 Units/kg weekly × 4 and 300 Units/kg daily × 15), 4 subjects in the 600 Units/kg weekly EPOGEN® group (5%) and no subjects in the 300 Units/kg daily group had a thrombotic vascular event during the study period.

In a study examining the use of Epoetin alfa in 182 patients scheduled for coronary artery bypass graft surgery, 23% of patients treated with Epoetin alfa and 29% treated with placebo experienced thrombotic/vascular events. There were 4 deaths among the Epoetin alfa-treated patients that were

associated with a thrombotic/vascular event. A causative role of Epoetin alfa cannot be excluded (see WARNINGS).

OVERDOSAGE

The maximum amount of EPOGEN® that can be safely administered in single or multiple doses has not been determined. Doses of up to 1500 Units/kg TIW for 3 to 4 weeks have been administered without any direct toxic effects of EPOGEN® itself.[6] Therapy with EPOGEN® can result in polycythemia if the hematocrit is not carefully monitored and the dose appropriately adjusted. If the suggested target range is exceeded, EPOGEN® may be temporarily withheld until the hematocrit returns to the suggested target range; EPOGEN® therapy may then be resumed using a lower dose (see DOSAGE AND ADMINISTRATION). If polycythemia is of concern, phlebotomy may be indicated to decrease the hematocrit.

DOSAGE AND ADMINISTRATION

Chronic Renal Failure Patients

Starting doses of EPOGEN® over the range of 50 to 100 Units/kg TIW have been shown to be safe and effective in increasing hematocrit and eliminating transfusion dependency in patients with CRF (see CLINICAL EXPERIENCE). The dose of EPOGEN® should be reduced as the hematocrit approaches 36% or increases by more than 4 points in any 2-week period. The dosage of EPOGEN® must be individualized to maintain the hematocrit within the suggested target range. At the physician's discretion, the suggested target hematocrit range may be expanded to achieve maximal patient benefit.

EPOGEN® may be given either as an IV or SC injection. In patients on hemodialysis, EPOGEN® usually has been administered as an IV bolus TIW. While the administration of EPOGEN® is independent of the dialysis procedure, EPOGEN® may be administered into the venous line at the end of the dialysis procedure to obviate the need for additional venous access. In patients with CRF not on dialysis, EPOGEN® may be given either as an IV or SC injection.

Patients who have been judged competent by their physicians to self-administer EPOGEN® without medical or other supervision may give themselves either an IV or SC injection. The table below provides general therapeutic guidelines for patients with CRF:

Starting Dose:	50 to 100 Units/kg TIW; IV or SC
Reduce Dose When:	1. Hct. approaches 36% or, 2. Hct. increases > 4 points in any 2-week period
Increase Dose If:	Hct. does not increase by 5 to 6 points after 8 weeks of therapy, and hct. is below suggested target range
Maintenance Dose:	Individually titrate
Suggested Target Hct. Range:	30% to 36%

During therapy, hematological parameters should be monitored regularly (see LABORATORY MONITORING).

Pre-therapy Iron Evaluation: Prior to and during EPOGEN® therapy, the patient's iron stores, including transferrin saturation (serum iron divided by iron-binding capacity) and serum ferritin, should be evaluated. Transferrin saturation should be at least 20%, and ferritin should be at least 100 ng/mL. Virtually all patients will eventually require supplemental iron to increase or maintain transferrin saturation to levels that will adequately support erythropoiesis stimulated by EPOGEN®.

Dose Adjustment: Following EPOGEN® therapy, a period of time is required for erythroid progenitors to mature and be released into circulation resulting in an eventual increase in hematocrit. Additionally, red blood cell survival time affects hematocrit and may vary due to uremia. As a result, the time required to elicit a clinically significant change in hematocrit (increase or decrease) following any dose adjustment may be 2 to 6 weeks.

Dose adjustment should not be made more frequently than once a month, unless clinically indicated. After any dose adjustment, the hematocrit should be determined twice weekly for at least 2 to 6 weeks (see LABORATORY MONITORING).

- If the hematocrit is increasing and approaching 36%, the dose should be reduced to maintain the suggested target hematocrit range. If the reduced dose does not stop the rise in hematocrit, and it exceeds 36%, doses should be temporarily withheld until the hematocrit begins to decrease, at which point therapy should be reinitiated at a lower dose.
- At any time, if the hematocrit increases by more than 4 points in a 2-week period, the dose should be immediately decreased. After the dose reduction, the hematocrit should be monitored twice weekly for 2 to 6 weeks, and further dose adjustments should be made as outlined in MAINTENANCE DOSE.
- If a hematocrit increase of 5 to 6 points is not achieved after an 8-week period and iron stores are adequate (see DELAYED OR DIMINISHED RESPONSE), the dose of EPOGEN® may be incrementally increased. Further increases may be made at 4 to 6 week intervals until the desired response is attained.

Maintenance Dose: The maintenance dose must be individualized for each patient on dialysis. In the US Phase 3 multicenter trial in patients on hemodialysis, the median maintenance dose was 75 Units/kg TIW, with a range from 12.5 to 525 Units/kg TIW. Almost 10% of the patients required a dose of 25 Units/kg, or less, and approximately 10% of the patients required more than 200 Units/kg TIW to maintain their hematocrit in the suggested target range.

If the hematocrit remains below or falls below the suggested target range, iron stores should be re-evaluated. If the transferrin saturation is less than 20%, supplemental iron should be administered. If the transferrin saturation is greater than 20%, the dose of EPOGEN® may be increased. Such dose increases should not be made more frequently than once a month, unless clinically indicated, as the response time of the hematocrit to a dose increase can be 2 to 6 weeks. Hematocrit should be measured twice weekly for 2 to 6 weeks following dose increases. In patients with CRF not on dialysis, the maintenance dose must also be individualized. EPOGEN® doses of 75 to 150 Units/kg per week have been shown to maintain hematocrits of 36% to 38% for up to 6 months.

Delayed or Diminished Response: Over 95% of patients with CRF responded with clinically significant increases in hematocrit, and virtually all patients were transfusion-independent within approximately 2 months of initiation of EPOGEN® therapy.

If a patient fails to respond or maintain a response, other etiologies should be considered and evaluated as clinically indicated (see PRECAUTIONS for discussion of delayed or diminished response).

Zidovudine-treated HIV-Infected Patients

Prior to beginning EPOGEN®, it is recommended that the endogenous serum erythropoietin level be determined (prior to transfusion). Available evidence suggests that patients receiving zidovudine with endogenous serum erythropoietin levels > 500 mUnits/mL are unlikely to respond to therapy with EPOGEN®.

Starting Dose: For patients with serum erythropoietin levels ≤ 500 mUnits/mL who are receiving a dose of zidovudine ≤ 4200 mg/week, the recommended starting dose of EPOGEN® is 100 Units/kg as an IV or SC injection TIW for 8 weeks.

Increase Dose: During the dose adjustment phase of therapy, the hematocrit should be monitored weekly. If the response is not satisfactory in terms of reducing transfusion requirements or increasing hematocrit after 8 weeks of therapy, the dose of EPOGEN® can be increased by 50 to 100 Units/kg TIW. Response should be evaluated every 4 to 8 weeks thereafter and the dose adjusted accordingly by 50 to 100 Units/kg increments TIW. If patients have not responded satisfactorily to an EPOGEN® dose of 300 Units/kg TIW, it is unlikely that they will respond to higher doses of EPOGEN®.

Maintenance Dose: After attainment of the desired response (ie, reduced transfusion requirements or increased hematocrit), the dose of EPOGEN® should be titrated to maintain the response based on factors such as variations in zidovudine dose and the presence of intercurrent infectious or inflammatory episodes. If the hematocrit exceeds 40%, the dose should be discontinued until the hematocrit drops to 36%. The dose should be reduced by 25% when treatment is resumed and then titrated to maintain the desired hematocrit.

Cancer Patients on Chemotherapy

Baseline endogenous serum erythropoietin levels varied among patients in these trials with approximately 75% (n = 83/110) having endogenous serum erythropoietin levels < 132 mUnits/mL, and approximately 4% (n = 4/110) of patients having endogenous serum erythropoietin levels > 500 mUnits/mL. In general, patients with lower baseline serum erythropoietin levels responded more vigorously to EPOGEN® than patients with higher erythropoietin levels. Although no specific serum erythropoietin level can be stipulated above which patients would be unlikely to respond to EPOGEN® therapy, treatment of patients with grossly elevated serum erythropoietin levels (eg, > 200 mUnits/mL) is not recommended. The hematocrit should be monitored on a weekly basis in patients receiving EPOGEN® therapy until hematocrit becomes stable.

Starting Dose: The recommended starting dose of EPOGEN® is 150 Units/kg SC TIW.

Dose Adjustment: If the response is not satisfactory in terms of reducing transfusion requirements or increasing hematocrit after 8 weeks of therapy, the dose of EPOGEN® can be increased up to 300 Units/kg TIW. If patients have not responded satisfactorily to an EPOGEN® dose of 300 Units/kg TIW, it is unlikely that they will respond to higher doses of EPOGEN®. If the hematocrit exceeds 40%, the dose of EPOGEN® should be withheld until the hematocrit falls to 36%. The dose of EPOGEN® should be reduced by 25% when treatment is resumed and titrated to maintain the desired hematocrit. If the initial dose of EPOGEN® includes a very rapid hematocrit response (eg, an increase of more than 4 percentage points in any 2-week period), the dose of EPOGEN® should be reduced.

Surgery Patients

Prior to initiating treatment with EPOGEN® a hemoglobin should be obtained to establish that it is > 10 to ≤ 13 g/dL.[14] The recommended dose of EPOGEN® is 300 Units/kg/day subcutaneously for 10 days before surgery, on the day of surgery, and for 4 days after surgery.

An alternate dose schedule is 600 Units/kg EPOGEN® subcutaneously in once weekly doses (21, 14, and 7 days before surgery) plus a fourth dose on the day of surgery.[15]

All patients should receive adequate iron supplementation. Iron supplementation should be initiated no later than the beginning of treatment with EPOGEN® and should continue throughout the course of therapy.

PREPARATION AND ADMINISTRATION OF EPOGEN®

1. Do not shake. It is not necessary to shake EPOGEN®. Prolonged vigorous shaking may denature any glycoprotein, rendering it biologically inactive.
2. Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration. Do not use any vials exhibiting particulate matter or discoloration.
3. Using aspetic techniques, attach a sterile needle to a sterile syringe. Remove the flip top from the vial containing EPOGEN®, and wipe the septum with a disinfectant. Insert the needle into the vial, and withdraw into the syringe an appropriate volume of solution.
4. Single-dose 1 mL vial contains no preservative. Use one dose per vial; do not re-enter the vial. Discard unused portions.

   Multidose 1 mL and 2 mL vials contain preservative. Store at 2° to 8°C after initial entry and between doses. Discard 21 days after initial entry.
5. Do not dilute or administer in conjunction with other drug solutions. However, at the time of SC administration, preservative-free EPOGEN® from single-use vials may be admixed in a syringe with bacteriostatic 0.9% sodium chloride injection, USP, with benzyl alcohol 0.9% (bacteriostatic saline) at a 1:1 ratio using aseptic technique. The benzyl alcohol in the bacteriostatic saline acts as a local anesthetic which may ameliorate SC injection site discomfort. Admixing is not necessary when using the multidose vials of EPOGEN® containing benzyl alcohol.

HOW SUPPLIED

EPOGEN®, containing Epoetin alfa, is available in the following packages:

1 mL Single-dose, Preservative-free Solution
- 2000 Units/mL (NDC 55513-126-10)
- 3000 Units/mL (NDC 55513-267-10)
- 4000 Units/mL (NDC 55513-148-10)
- 10,000 Units/mL (NDC 55513-144-10)

Supplied in cartons containing 10 single-dose vials.

2 mL Multidose, Preserved Solution
- 10,000 Units/mL (NDC 55513-283-10)

1 mL Multidose, Preserved Solution
- 20,000 Units/mL (NDC 55513-478-10)

Supplied in cartons containing 10 multidose vials.

STORAGE

Store at 2° to 8°C (36° to 46°F). Do not freeze or shake.

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Epogen—Cont.

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AMGEN®
Manufactured by:
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91320-1789
Issue Date: 12/23/96

EPOGEN®(Epoetin alfa)
Information for Home Dialysis Patients

AMGEN®
EPOGEN®
(RECOMBINANT EPOETIN ALFA)

What is EPOGEN® and how does it work?

EPOGEN® is a copy of human erythropoietin, a hormone produced primarily by healthy kidneys. EPOGEN® replaces the erythropoietin that the failed kidneys can no longer produce, and signals the bone marrow to make the oxygen-carrying red blood cells once again. EPOGEN® is produced in mammalian cells that have been genetically altered by the addition of gene for the natural substance erythropoietin.

How should I take EPOGEN®?

In those situations where your doctor has determined that you, as a home dialysis patient, can self-administer EPOGEN®, you will receive instruction on how much EPOGEN® to use, how to inject it, how often you should inject it, and how you should dispose of the unused portions of each vial.

You will be instructed to monitor your blood pressure carefully everyday and to report any changes outside of the guidelines that your doctor has given you. When the number of red blood cells increases, your blood pressure can also increase, so your doctor may prescribe some new or additional blood pressure medication. Be sure to follow your doctor's orders. You may also be instructed to have certain laboratory tests, such as additional hematocrit or iron level measurements, done more frequently. You may be asked to report these tests to your doctor or dialysis center. Also, your doctor may prescribe additional iron for you to take. Be sure to comply with your doctor's orders.

Continue to check your access, as your doctor or nurse has shown you, to make sure it is working. Be sure to let your health care professional know right away if there is a problem.

Allergy to EPOGEN®

Patients occasionally experience redness, swelling, or itching at the site of injection of EPOGEN®. This may indicate an allergy to the components of EPOGEN®, or it may indicate a local reaction. If you have a local reaction, consult your doctor. A potentially more serious reaction would be a generalized allergy to EPOGEN®, which could cause a rash over the whole body, shortness of breath, wheezing, reduction in blood pressure, fast pulse, or sweating. Severe cases of generalized allergy may be life-threatening. If you think you are having a generalized allergic reaction, stop taking EPOGEN® and notify a doctor or emergency medical personnel imediately.

How will I know if EPOGEN® is working?

The effectiveness of EPOGEN® is measured by the increase in hematocrit (the amount of red blood cells in the blood) that results from EPOGEN® therapy. The rise in hematocrit is not immediate. It usually takes about 2 to 6 weeks before the hematocrit starts to rise. The amount of time it takes, and the dose of EPOGEN® that is needed to make the hematocrit increase, varies from patient to patient.

What is the most important information I should know about EPOGEN® and CHRONIC RENAL FAILURE?

EPOGEN® has been prescribed for you by your doctor because you:

1. Have anemia due to your kidney disease.
2. Are able to dialyze at home.
3. Have been determined to be able to administer EPOGEN® without direct medical or other supervision.

A lack of energy or feeling of tiredness is the major symptom of anemia. Additional symptoms include shortness of breath, chest pain, and feeling cold all the time. The reason for these symptoms is that there is a lack of red blood cells. Red blood cells carry oxygen, which is important for all of the body's functions. When there are fewer red blood cells, the body does not get all the oxygen it needs.

Kidneys remove toxins from the blood; they also measure the amount of oxygen in the blood. If there is not enough oxygen, the kidneys will produce a hormone called erythropoietin. Erythropoietin is released into the bloodstream and travels to the bone marrow where red blood cells are made. Erythropoietin signals the bone marrow to make more oxygen-carrying red blood cells.

As the kidneys fail, they stop cleansing toxins from your body. They also make less erythropoietin than they should. Therefore, the bone marrow does not receive a strong-enough signal to make the oxygen-carrying red blood cells. Fewer red blood cells are produced so the muscles, brain, and other parts of the body do not get the oxygen they need to function properly.

Most patients treated with EPOGEN® no longer need blood transfusions. However, certain medical conditions, or unexpected blood loss, may result in the need for a transfusion.

What do I need to know if I am giving myself EPOGEN® injections?

When you receive your EPOGEN® from the dialysis center, doctor's office or home dialysis supplier, always check to see that:

1. The name EPOGEN® appears on the carton and vial label.
2. You will be able to use EPOGEN® before the expiration date stamped on the package.

The EPOGEN® solution in the vial should always be clear and colorless. Do not use EPOGEN® if the contents of the vial appear discolored or cloudy, or if the vial appears to contain lumps, flakes, or particles. In addition, if the vial has been shaken vigorously, the solution may appear to be frothy and should not be used. Therefore, care should be taken not to shake the EPOGEN® vial vigorously before use.

Single Use Vials–S

If you have been prescribed EPOGEN® vials for single use, your vial will have a capital "S" with a number next to it identifying the concentration of EPOGEN® in the vial, printed in a colored dot on the front left side of the label (for example, "S2" identifies a single use vial with 2000 Units/mL). Single use means the vial cannot be used more than once, and any unused portion of the vial should be discarded as directed by your doctor or dialysis center.

Multidose Use Vials–M

If you have been prescribed EPOGEN® Multidose vials, your vial will have a capital "M" with a number under it identifying the concentration of EPOGEN® in the vial, printed in a colored dot on the front left side of the label (for example, "M10" identifies a Multidose vial with 10,000 Units/mL). Multidose EPOGEN® can be used to inject multiple doses as prescribed by your doctor, and may be stored in the refrigerator (but not the freezing compartment) between doses for up to 21 days. Follow your doctor's or dialysis center's instructions on what to do with the used vials.

How should I store EPOGEN®?

EPOGEN® should be stored in the refrigerator, but not in the freezing compartment. Do not let the vial freeze and do not leave it in direct sunlight. Do not use a vial of EPOGEN® that has been frozen or after the expiration date that is stamped on the label. If you have any questions about the safety of a vial of EPOGEN® that has been subjected to temperature extremes, be sure to check with your dialysis unit staff.

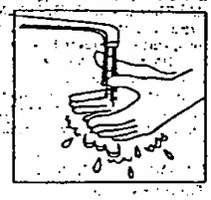
Always use the correct syringe

Your doctor has instructed you on how to give yourself the correct dosage of EPOGEN®. This dosage will usually be measured in Units per milliliter or CCs. It is important to use a syringe that is marked in tenths of milliliters (for example, 0.2 mL or CC). Failure to use the proper syringe can lead to a mistake in dosage, and you may receive too much or too little EPOGEN®. Too little EPOGEN® may not be effective in increasing your hematocrit, and too much EPOGEN® may lead to a hematocrit that is too high. Only use disposable syringes and needles as they do not require sterilization; they should be used once and disposed of as instructed by your doctor.

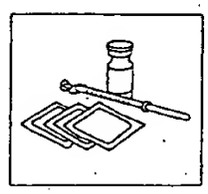
IMPORTANT: TO HELP AVOID CONTAMINATION AND POSSIBLE INFECTION, FOLLOW THESE INSTRUCTIONS EXACTLY.

PREPARING THE DOSE

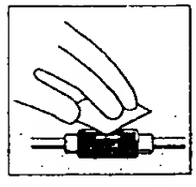
1. Wash your hands thoroughly with soap and water before preparing the medication.

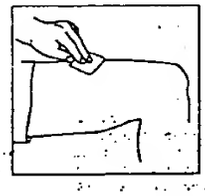


2. Check the date on the EPOGEN® vial to be sure that the drug has not expired.
3. Remove the vial of EPOGEN® from the refrigerator and allow it to reach room temperature. Unless you are using a Multidose vial, each EPOGEN® vial is designed to be used only once. It is not necessary to shake EPOGEN®. Prolonged vigorous shaking may damage the product. Assemble the other supplies you will need for your injection.

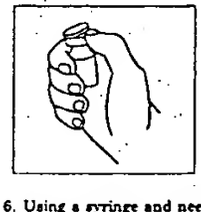


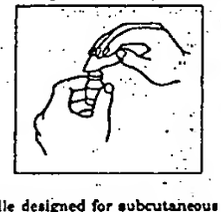
4. Hemodialysis patients should wipe off the venous port of the hemodialysis tubing with an antiseptic swab. Peritoneal dialysis patients should cleanse the skin with an antiseptic swab where the injection is to be made.



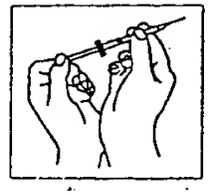


5. Flip off the red protective cap but do not remove the gray rubber stopper. Wipe the top of the gray rubber stopper with an antiseptic swab.



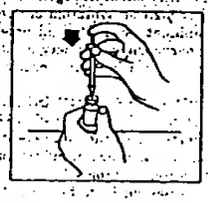


6. Using a syringe and needle designed for subcutaneous injection, draw air into the syringe by pulling back on the plunger. The amount of air should be equal to your EPOGEN® dose.

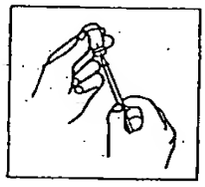


7. Carefully remove the needle cover. Put the needle through the gray rubber stopper of the EPOGEN® vial.

8. Push the plunger in to discharge air into the vial. The air injected into the vial will allow EPOGEN® to be easily withdrawn into the syringe.



9. Turn the vial and syringe upside down in one hand. Be sure the tip of the needle is in the EPOGEN® solution. Your other hand will be free to move the plunger. Draw back on the plunger slowly to draw the correct dose of EPOGEN® into the syringe.

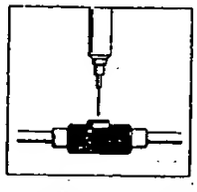


10. Check for air bubbles. The air is harmless, but too large an air bubble will reduce the EPOGEN® dose. To remove air bubbles, gently tap the syringe to move the air bubbles to the top of the syringe, then use the plunger to push the solution and the air back into the vial. Then remeasure your correct dose of EPOGEN®.
11. Double check your dose. Remove the needle from the vial. Do not lay the syringe down or allow the needle to touch anything.

INJECTING THE DOSE

Patients on home hemodialysis using the intravenous injection route:

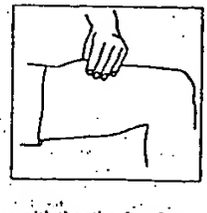
1. Insert the needle of the syringe into the previously cleansed venous port and inject the EPOGEN®.



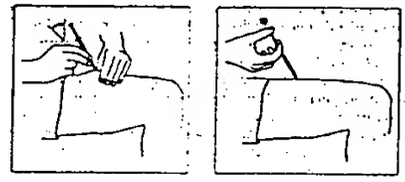
2. Remove the syringe and dispose of the whole unit. ***Use the disposable syringe only once.*** Dispose of syringes and needles as directed by your doctor, by following these simple steps:
   - Place all used needles and syringes in a hard plastic container with a screw-on-cap, or a metal container with a plastic lid, such as a coffee can properly labeled as to content. If a metal container is used, cut a small hole in the plastic lid and tape the lid to the metal container. If a hard-plastic container is used, always screw the cap on tightly after each use. When the container is full, tape around the cap or lid, and dispose of according to your doctor's instructions.
   - Do not use glass or clear plastic containers, or any container that will be recycled or returned to a store.
   - Always store the container out of the reach of children.
   - Please check with your doctor, nurse, or pharmacist for other suggestions. There may be special state and local laws that they will discuss with you.

Patients on home peritoneal dialysis or home hemodialysis using the subcutaneous route:

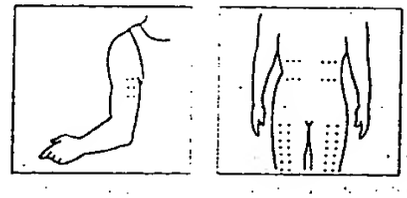
1. With one hand, stabilize the previously cleansed skin by spreading it or by pinching up a large area with your free hand.



2. Hold the syringe with the other hand, as you would a pencil. Double check that the correct amount of EPOGEN® is in the syringe. Insert the needle straight into the skin (90 degree angle). Pull the plunger back slightly. If blood comes into the syringe, do not inject EPOGEN®, as the needle has entered a blood vessel; withdraw the syringe and inject at a different site. Inject the EPOGEN® by pushing the plunger all the way down.



3. Hold an antiseptic swab near the needle and pull the needle straight out of the skin. Press the antiseptic swab over the injection site for several seconds.
4. ***Use the disposable syringe only once.*** Dispose of syringes and needles as directed by your doctor, by following these simple steps:
   - Place all used needles and syringes in a hard plastic container with a screw-on-cap, or a metal container with a plastic lid, such as a coffee can properly labeled as to content. If a metal container is used, cut a small hole in the plastic lid and tape the lid to the metal container. If a hard-plastic container is used, always screw the cap on tightly after each use. When the container is full, taper around the cap or lid, and dispose of according to your doctor's instructions.
   - Do not use glass or clear plastic containers, or any container that will be recycled or returned to a store.
   - Always store the container out of the reach of children.
   - Please check with your doctor, nurse, or pharmacist for other suggestions. There may be special state and local laws that they will discuss with you.
5. Always change the site for each injection as directed. Occassionally a problem may develop at the injection site. If you notice a lump, swelling, or bruising that doesn't go away, contact your doctor. You may wish to record the site just used so that you can keep track.



USAGE IN PREGNANCY

If you are pregnant or nursing a baby, consult your doctor before using EPOGEN®.

IMPORTANT NOTES

Since you are a home dialysis patient and your doctor allows you to self-administer EPOGEN®, please note the following:

1. Always follow the instructions of your doctor concerning the dosage and administration of EPOGEN®. Do not change the dose or instructions for administration of EPOGEN® without consulting your doctor.
2. Your doctor will tell you what to do if you miss a dose of EPOGEN®. Always keep a spare syringe and needle on hand.
3. Always consult your doctor if you notice anything unusual about your condition or your use of EPOGEN®.

AMGEN®
Manufactured by:
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91320-1789

Issue Date: 11/14/96 US EPO FI Copy Rev

P30035D 25M/1-97
Shown in Product Identification Guide, page 304

INFERGEN®
(Interferon alfacon-1)

DESCRIPTION

Interferon alfacon-1 is a recombinant non-naturally occurring type-I interferon. The 166-amino acid sequence of Interferon alfacon-1 was derived by scanning the sequences of several natural interferon alpha subtypes and assigning the most frequently observed amino acid in each corresponding position.[1] Four additional amino acid changes were made to facilitate the molecular construction, and a corresponding synthetic DNA sequence was constructed using E. coli gene synthesis methodology. Interferon alfacon-1 differs from interferon alfa-2 at 20/166 amino acid (88% homology). In comparison with interferon beta it shows identity at over 30% of the amino acid positions. Interferon alfacon-1 is produced in *Escherichia coli (E coli)* cells that have been genetically altered by insertion of a synthetically constructed sequence that codes for Interferon alfacon-1. Prior to final purification, Interferon alfacon-1 is allowed to oxidize to its native state, and its final purity is achieved by sequential passage over a series of chromatography columns. This protein has a molecular weight of 19,434 daltons. Infergen® is the Amgen Inc. trademark for Interferon alfacon-1.

Infergen is a sterile, clear, colorless, preservative-free liquid formulated with 100 mM sodium chloride and 15 mM sodium phosphate at pH 7.0 ± 0.2. The product is available in single-use vials and prefilled syringes containing 9 mcg and 15 mcg Interferon alfacon-1 at a fill volume of 0.3 mL and 0.5 mL, respectively. Infergen vials and prefilled syringes contain 0.03 mg/mL of Interferon alfacon-1, 5.8 mg/mL sodium chloride, and 3.8 mg/mL sodium phosphate in Water for Injection, USP. The Infergen SingleJect™ prefilled syringe has a glass barrel and a 26 gauge, 5/8 inch needle. Infergen is to be administered undiluted by subcutaneous (SC) injection.

Formulation, filling, and packaging operations for Infergen are performed by Amgen Puerto Rico, a wholly-owned subsidiary of Amgen Inc.

CLINICAL PHARMACOLOGY

General

Interferons are a family of naturally occurring small protein molecules with molecular weights of 15,000 to 21,000 daltons that are produced and secreted by cells in response to viral infections or to various synthetic and biological inducers. Two major classes of interferons have been identified (ie, type-I and type-II). Type-I interferons include a family of more than 25 interferon alphas as well as interferon beta and interferon omega. While all alpha interferons have similar biological effects, not all the activities are shared by each alpha interferon and, in many cases, the extent of activity varies substantially for each interferon subtype.

All type-I interferons share common biological activities generated by binding of interferon to the cell-surface receptor, leading to the production of several interferon-stimulated gene products. Type-I interferons induce pleiotropic biologic responses which include antiviral, antiproliferative and immunomodulatory effects, regulation of cell surface major histocompatibility antigen (HLA class I and class II) expression and regulation of cytokine expression. Examples of interferon-stimulated gene products include 2'5' oligoadenylate synthetase (2'5' OAS) and β-2 microglobulin.

The antiviral, antiproliferative, NK cell activation, and gene-induction activities of Infergen have been compared with other recombinant alfa interferons in *in vitro* assays and have demonstrated similar ranges of activity. Infergen exhibited at least five times higher specific activity in more than Interferon alfa-2a and Interferon alfa-2b.[2] Comparison of Infergen with a WHO international potency standard for recombinant interferon alfa (83/514) revealed that the specific activity of Infergen in both an *in vitro* antiviral cytopathic effect assay and an antiproliferative assay was $> 10^9$ U/mg. However, correlation between *in vitro* activity and clinical activity of any interferon is unknown.

Pharmacokinetics and Pharmacodynamics

The pharmacokinetic properties of Infergen have not been evaluated in patients with chronic hepatitis C. Pharmacokinetic profiles were evaluated in normal, healthy volunteer subjects after SC injection of 1, 3, or 9 mcg Interferon alfacon-1. Plasma levels of Infergen after SC administration of any dose were too low to be detected by either ELISA or by inhibition of viral cytopathic effect. However, analysis of Infergen-induced cellular products (induction of 2'5' OAS and β-2 microglobulin) after treatment in these subjects revealed a statistically significant, dose-related increase in the area under the curve (AUC) for the levels of 2'5' OAS or β-2 microglobulin induced over time ($p < 0.001$ for all comparisons). Concentrations of 2'5' OAS were maximal at 24 hours after dosing, while serum levels of β-2 microglobulin appeared to reach a maximum 24 to 36 hours after dosing. The dose-response relationships observed for 2'5' OAS and β-2 microglobulin were indicative of biological activity after SC administration of 1 to 9 mcg Infergen.

Preclinical Experience

All interferons have been shown to be highly species specific. Antiviral activity of Infergen was observed in the rhesus monkey LLC cell line and golden Syrian hamster BHK cell line. Antiviral activity of Infergen in the golden Syrian hamster was confirmed further *in vivo*.[3] Pharmacokinetic studies of Infergen in golden Syrian hamsters and rhesus monkeys demonstrated rapid absorption following SC injec-

Continued on next page

Administer ORTHOCLONE OKT3 as a single intravenous (bolus) injection in less than one minute. Do not administer by intravenous infusion or in conjunction with other drug solutions.

HOW SUPPLIED

ORTHOCLONE OKT3 is supplied as a sterile solution in packages of 5 ampules (NDC 59676-101-01). Each 5 mL ampule contains 5 mg of muromonab-CD3.
Storage: Store in a refrigerator at 2° to 8°C (36° to 46°F). DO NOT FREEZE OR SHAKE.

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PROCRIT®
EPOETIN ALFA
PROCRIT registered trademark of distributor
FOR INJECTION

DESCRIPTION

Erythropoietin is a glycoprotein which stimulates red blood cell production. It is produced in the kidney and stimulates the division and differentiation of committed erythroid progenitors in the bone marrow. PROCRIT (Epoetin alfa), a 165 amino acid glycoprotein manufactured by recombinant DNA technology, has the same biological effects as endogenous erythropoietin.[1] It has a molecular weight of 30,400 daltons and is produced by mammalian cells into which the human erythropoietin gene has been introduced. The product contains the identical amino acid sequence of isolated natural erythropoietin.
PROCRIT is formulated as a sterile, colorless, liquid in an isotonic sodium chloride/sodium citrate buffered solution for intravenous (IV) or subcutaneous (SC) administration.
Single-Dose, Preservative-Free Vial: 1 mL (2,000, 3,000, 4,000 or 10,000 Units/mL). Each 1 mL of solution contains 2,000, 3,000, 4,000 or 10,000 Units of Epoetin alfa, 2.5 mg Albumin (Human), 5.8 mg sodium citrate, 5.8 mg sodium chloride, and 0.06 mg citric acid in Water for Injection, USP (pH 6.9±0.3). This formulation contains no preservative.
Single-Dose, Preservative-Free Vial: 1 mL (40,000 Units/mL). Each 1 mL of solution contains 40,000 Units of Epoetin alfa, 2.5 mg Albumin (Human), 1.164 mg sodium phosphate monobasic monohydrate, 1.766 mg sodium phosphate dibasic anhydrate, 0.696 mg sodium citrate, 5.78 mg sodium chloride, and 6.8 mcg citric acid in Water for Injection, USP (pH 6.9±0.3). This formulation contains no preservative.
Multidose, Preserved Vial: 2 mL (20,000 Units, 10,000 Units/mL). Each 1 mL of solution contains 10,000 Units of Epoetin alfa, 2.5 mg Albumin (Human), 1.3 mg sodium citrate, 8.2 mg sodium chloride, 0.11 mg citric acid, and 1% benzyl alcohol as preservative in Water for Injection, USP (pH 6.1±0.3).
Multidose, Preserved Vial: 1 mL (20,000 Units/mL). Each 1 mL of solution contains 20,000 Units of Epoetin alfa, 2.5 mg Albumin (Human), 1.3 mg sodium citrate, 8.2 mg sodium chloride, 0.11 mg citric acid, and 1% benzyl alcohol as preservative in Water for Injection, USP (pH 6.1±0.3).

CLINICAL PHARMACOLOGY

Chronic Renal Failure Patients

Endogenous production of erythropoietin is normally regulated by the level of tissue oxygenation. Hypoxia and anemia generally increase the production of erythropoietin, which in turn stimulates erythropoiesis.[2] In normal subjects, plasma erythropoietin levels range from 0.01 to 0.03 Units/mL[2,3] and increase up to 100- to 1000-fold during hypoxia or anemia.[2,3] In contrast, in patients with chronic renal failure (CRF), production of erythropoietin is impaired, and this erythropoietin deficiency is the primary cause of their anemia.[3,4]
Chronic renal failure is the clinical situation in which there is a progressive and usually irreversible decline in kidney function. Such patients may manifest the sequelae of renal dysfunction, including anemia, but do not necessarily require regular dialysis. Patients with end-stage renal disease (ESRD) are those patients with CRF who require regular dialysis or kidney transplantation for survival.
PROCRIT has been shown to stimulate erythropoiesis in anemic patients with CRF, including both patients on dialysis and those who do not require regular dialysis.[4-13] The first evidence of a response to the three times weekly (T.I.W.) administration of PROCRIT is an increase in the reticulocyte count within 10 days, followed by increases in the red cell count, hemoglobin, and hematocrit, usually within 2-6 weeks.[4,5] Because of the length of time required for erythropoiesis — several days for erythroid progenitors to mature and be released into the circulation — a clinically significant increase in hematocrit is usually not observed in less than 2 weeks and may require up to 6 weeks in some patients. Once the hematocrit reaches the suggested target range (30-36%), that level can be sustained by PROCRIT therapy in the absence of iron deficiency and concurrent illnesses.
The rate of hematocrit increase varies between patients and is dependent upon the dose of PROCRIT, within a therapeutic range of approximately 50-300 Units/kg (T.I.W.).[4] A greater biologic response is not observed at doses exceeding 300 Units/kg (T.I.W.).[6] Other factors affecting the rate and extent of response include availability of iron stores, the baseline hematocrit, and the presence of concurrent medical problems.

Zidovudine-treated HIV-Infected Patients

Responsiveness to PROCRIT in HIV-infected patients is dependent upon the endogenous serum erythropoietin level prior to treatment. Patients with endogenous serum erythropoietin levels ≤ 500 mUnits/mL, and who are receiving a dose of zidovudine ≤ 4,200 mg/week, may respond to PROCRIT therapy. Patients with endogenous serum erythropoietin levels > 500 mUnits/mL do not appear to respond to PROCRIT therapy. In a series of four clinical trials involving 255 patients, 60% to 80% of HIV-infected patients treated with zidovudine had endogenous serum erythropoietin levels ≤ 500 mUnits/mL.
Response to PROCRIT in zidovudine-treated, HIV-infected patients is manifested by reduced transfusion requirements and increased hematocrit.

Cancer Patients on Chemotherapy

Anemia in cancer patients may be related to the disease itself or the effect of concomitantly administered chemotherapeutic agents. PROCRIT has been shown to increase hematocrit and decrease transfusion requirements after the first month of therapy (months 2 and 3), in anemic cancer patients undergoing chemotherapy.
A series of clinical trials enrolled 131 anemic cancer patients who were receiving cyclic cisplatin- or non cisplatin-containing chemotherapy. Endogenous baseline serum erythropoietin levels varied among patients in these trials with approximately 75% (N=83/110) having endogenous serum erythropoietin levels ≤ 132 mUnits/mL, and approximately 4% (N=4/110) of patients having endogenous serum erythropoietin levels > 500 mUnits/mL. In general, patients with lower baseline serum erythropoietin levels responded more vigorously to PROCRIT than patients with higher baseline erythropoietin levels. Although no specific serum erythropoietin level can be stipulated above which patients would be unlikely to respond to PROCRIT therapy, treatment of patients with grossly elevated serum erythropoietin levels (e.g., > 200 mUnits/mL) is not recommended.

Pharmacokinetics

Intravenously administered PROCRIT is eliminated at a rate consistent with first order kinetics with a circulating half-life ranging from approximately 4 to 13 hours in patients with CRF. Within the therapeutic dose range, detectable levels of plasma erythropoietin are maintained for at least 24 hours.[7] After subcutaneous administration of PROCRIT to patients with CRF, peak serum levels are achieved within 5-24 hours after administration and decline slowly thereafter. There is no apparent difference in half-life between patients not on dialysis whose serum creatinine levels were greater than 3, and patients maintained on dialysis.
In normal volunteers, the half-life of intravenously administered PROCRIT is approximately 20% shorter than the half-life in CRF patients. The pharmacokinetics of PROCRIT have not been studied in HIV-infected patients. It has been demonstrated in normal volunteers that the 10,000 U/mL citrate-buffered Epoetin alfa formulation and the 40,000 U/mL phosphate-buffered Epoetin alfa formulation are bioequivalent after subcutaneous administration of single 750 Units/kg doses. The C_{max} and $t_{\frac{1}{2}}$ after administration of the phosphate buffered Epoetin alfa formulation were 1.80 ± 0.7 U/mL and 19.0 ± 5.9 hours (mean ± SD), respectively. The corresponding mean ± SD values for the citrate-buffered Epoetin alfa formulation were 2 ± 0.9 U/mL and 16.3 ± 3.9 hours. There was minimal accumulation in serum after two weekly 750 Units/kg subcutaneous doses of Epoetin alfa.

INDICATIONS AND USAGE

Treatment of Anemia of Chronic Renal Failure Patients

PROCRIT is indicated in the treatment of anemia associated with chronic renal failure, including patients on dialysis (end-stage renal disease) and patients not on dialysis. PROCRIT is indicated to elevate or maintain the red blood cell level (as manifested by the hematocrit or hemoglobin determinations) and to decrease the need for transfusions in these patients.
Non-dialysis patients with symptomatic anemia considered for therapy should have a hematocrit less than 30%.
PROCRIT is not intended for patients who require immediate correction of severe anemia. PROCRIT may obviate the need for maintenance transfusions but is not a substitute for emergency transfusion.
Prior to initiation of therapy, the patient's iron stores should be evaluated. Transferrin saturation should be at least 20% and ferritin at least 100 ng/mL. Blood pressure should be adequately controlled prior to initiation of PROCRIT therapy, and must be closely monitored and controlled during therapy.
PROCRIT should be administered under the guidance of a qualified physician (see "DOSAGE and ADMINISTRATION").

Treatment of Anemia in Zidovudine-treated HIV-Infected Patients

PROCRIT is indicated for the treatment of anemia related to therapy with zidovudine in HIV-infected patients. PROCRIT is indicated to elevate or maintain the red blood cell level (as manifested by the hematocrit or hemoglobin determinations) and to decrease the need for transfusions in these patients. PROCRIT is not indicated for the treatment of anemia in HIV-infected patients due to other factors such as iron or folate deficiencies, hemolysis or gastrointestinal bleeding, which should be managed appropriately.
PROCRIT, at a dose of 100 Units/kg three times per week, is effective in decreasing the transfusion requirement and increasing the red blood cell level of anemic, HIV-infected patients treated with zidovudine, when the endogenous serum erythropoietin level is ≤ 500 mUnits/mL and when patients are receiving a dose of zidovudine ≤ 4,200 mg/week.

Treatment of Anemia in Cancer Patients on Chemotherapy

PROCRIT is indicated for the treatment of anemia in patients with non-myeloid malignancies where anemia is due to the effect of concomitantly administered chemotherapy. PROCRIT is indicated to decrease the need for transfusions in patients who will be receiving concomitant chemotherapy for a minimum of 2 months. PROCRIT is not indicated for the treatment of anemia in cancer patients due to other factors such as iron or folate deficiencies, hemolysis or gastrointestinal bleeding which should be managed appropriately.

Reduction of Allogeneic Blood Transfusion in Surgery Patients

PROCRIT is indicated for the treatment of anemic patients (hemoglobin >10 to ≤13 g/dL) scheduled to undergo elective, noncardiac, nonvascular surgery to reduce the need for allogeneic blood transfusions.[14-15] PROCRIT is indicated for patients at high risk for perioperative transfusions with significant, anticipated blood loss. PROCRIT is not indicated for anemic patients who are willing to donate autologous blood. The safety of the perioperative use of PROCRIT has been studied only in patients who are receiving anticoagulant prophylaxis.

Clinical Experience: Response to PROCRIT

Chronic Renal Failure Patients

Response to PROCRIT was consistent across all studies. In the presence of adequate iron stores (see "Iron Evaluation"), the time to reach the target hematocrit is a function of the baseline hematocrit and the rate of hematocrit rise.
The rate of increase in hematocrit is dependent upon the dose of PROCRIT administered and individual patient variation. In clinical trials at starting doses of 50-150 Units/kg (T.I.W.), patients responded with an average rate of hematocrit rise of:

HEMATOCRIT INCREASE

STARTING DOSE (T.I.W. IV)	POINTS/DAY	POINTS/ 2 WEEKS
50 Units/kg	0.11	1.5
100 Units/kg	0.18	2.5
150 Units/kg	0.25	3.5

Over this dose range, approximately 95% of all patients responded with a clinically significant increase in hematocrit, and by the end of approximately 2 months of therapy virtually all patients were transfusion-independent. Changes in

Continued on next page

ocrit—Cont.

quality of life of patients treated with PROCRIT were ssed as part of a Phase III clinical trial.[5,8] Once the tar- hematocrit (32-38%) was achieved, statistically signifi- improvements were demonstrated for most quality of parameters measured, including energy and activity l, functional ability, sleep and eating behavior, health us, satisfaction with health, sex life, well-being, psycho- al effect, life satisfaction, and happiness. Patients also orted improvement in their disease symptoms. They wed a statistically significant increase in exercise capac- (VO_2 max), energy, and strength with a significant re- tion in aching, dizziness, anxiety, shortness of breath, scle weakness, and leg cramps.[8,17]

ients On Dialysis: Thirteen clinical studies were con- ted, involving intravenous administration to a total of 10 anemic patients on dialysis for 986 patient-years of OCRIT therapy. In the three largest of these clinical tri- the median maintenance dose necessary to maintain hematocrit between 30-36% was approximately 75 its/kg (T.I.W.). In the U.S. multicenter Phase III study, proximately 65% of the patients required doses of 100 its/kg (T.I.W.), or less, to maintain their hematocrit at ap- ximately 35%. Almost 10% of patients required a dose of Units/kg, or less, and approximately 10% required a dose nore than 200 Units/kg (T.I.W.) to maintain their hemat- it at this level.

nulticenter unit dose study was also conducted in 119 pa- nts receiving peritoneal dialysis who self-administered OCRIT subcutaneously for approximately 109 patient- ars of experience. Patients responded to PROCRIT ad- nistered subcutaneously in a manner similar to patients ceiving intravenous administration.[18]

tients With CRF Not Requiring Dialysis: Four clinical als were conducted in patients with CRF not on dialysis volving 181 patients treated with PROCRIT for approxi- tely 67 patient-years of experience. These patients re- onded to PROCRIT therapy in a manner similar to that served in patients on dialysis. Patients with CRF not on alysis demonstrated a dose-dependent and sustained in- ease in hematocrit when PROCRIT was administered by ther an intravenous (IV) or subcutaneous (SC) route, with milar rates of rise of hematocrit when PROCRIT was ad- inistered by either route. Moreover, PROCRIT doses of 75- 50 Units/kg per week have been shown to maintain hema- crits of 36-38% for up to six months. Correcting the ane- ia of progressive renal failure will allow patients to main active even though their renal function continues to crease.[19-21]

idovudine-treated HIV-infected Patients

ROCRIT has been studied in four placebo-controlled trials arolling 297 anemic (hematocrit < 30%) HIV-infected IDS) patients receiving concomitant therapy with zidovu- ne, (all patients were treated with Epoetin alfa manufac- ured by Amgen Inc.). In the subgroup of patients (89/125 ROCRIT, and 88/130 placebo) with prestudy endogenous erum erythropoietin levels ≤ 500 mUnits/mL PROCRIT educed the mean cumulative number of units of blood ransfused per patient by approximately 40%, as compared o the placebo group.[22] Among those patients who required ransfusions at baseline, 43% of patients treated with ROCRIT versus 18% of placebo-treated patients were ransfusion-independent during the second and third nonths of therapy. PROCRIT therapy also resulted in sig- ificant increases in hematocrit in comparison to placebo. When examining the results according to the weekly dose of idovudine received during Month 3 of therapy, there was a tatistically significant ($p < 0.003$) reduction in transfusion equirements in patients treated with PROCRIT (N=51) ompared to placebo-treated patients (N=54) whose mean weekly zidovudine dose was ≤ 4,200 mg/week.[22]

Approximately 17% of the patients with endogenous serum erythropoietin levels ≤ 500 mUnits/mL receiving PROCRIT n doses from 100-200 Units/kg three times weekly (T.I.W.) achieved a hematocrit of 38% without administration of transfusions or a significant reduction in zidovudine dose. In the subgroup of patients whose prestudy endogenous serum erythropoietin levels were > 500 mUnits/mL, PROCRIT therapy did not reduce transfusion requirements or increase hematocrit, compared to the corresponding responses in placebo-treated patients.

In a six month open-label PROCRIT study, patients responded with decreased transfusion requirements and sustained increases in hematocrit and hemoglobin with doses of PROCRIT up to 300 Units/kg (T.I.W.).[21-23]

Responsiveness to PROCRIT therapy may be blunted by intercurrent infectious/inflammatory episodes and by an increase in zidovudine dosage. Consequently, the dose of PROCRIT must be titrated based on these factors to maintain the desired erythropoietic response.

Cancer Patients on Chemotherapy

PROCRIT has been studied in a series of placebo-controlled, double-blind trials in a total of 131 anemic cancer patients. Within this group, 72 patients were treated with concomitant noncisplatin-containing chemotherapy regimens and 59 patients were treated with concomitant cisplatin-containing chemotherapy regimens. Patients were randomized to PROCRIT 150 Units/kg or placebo subcutaneously (T.I.W.) for 12 weeks.

PROCRIT therapy was associated with a significantly ($p<0.008$) greater hematocrit response than in the corresponding placebo-treated patients (see TABLE).[22]

HEMATOCRIT (%): MEAN CHANGE FROM BASELINE TO FINAL VALUE*

STUDY	PROCRIT	PLACEBO
Chemotherapy	7.6	1.3
Cisplatin	6.9	0.6

* Significantly higher in PROCRIT patients than in placebo patients ($p < 0.008$)

In the two types of chemotherapy studies [utilizing a PROCRIT dose of 150 Units/kg (T.I.W.)] the mean number of units of blood transfused per patient after the first month of therapy was significantly ($p < 0.02$) lower in patients treated with PROCRIT (0.71 units in Months 2, 3) than in corresponding placebo-treated patients (1.84 units in Months 2, 3). Moreover, the proportion of patients transfused during Months 2 and 3 of therapy combined was significantly ($p < 0.03$) lower in the patients treated with PROCRIT than in the corresponding placebo-treated patients (22% versus 43%).[22]

Comparable intensity of chemotherapy in the PROCRIT and placebo groups in the chemotherapy trials was suggested by a similar area under the neutrophil time curve in patients treated with PROCRIT and placebo-treated patients as well as by a similar proportion of patients in groups treated with PROCRIT and placebo-treated groups whose absolute neutrophil counts fell below 1,000 cells/μL. Available evidence suggests that patients with lymphoid and solid cancers respond equivalently to PROCRIT therapy, and that patients with or without tumor infiltration of the bone marrow respond equivalently to PROCRIT therapy.

Surgery Patients

PROCRIT has been studied in a placebo-controlled, double-blind trial enrolling 316 patients scheduled for major, elective orthopedic hip or knee surgery who were expected to require ≥2 units of blood and who were not able or willing to participate in an autologous blood donation program. Based on previous studies which demonstrated that pretreatment hemoglobin is a predictor of risk of receiving transfusion[16,24], patients were stratified into one of three groups based on their pretreatment hemoglobin [≤10 (n=2), >10 to ≤13 (n=96), and >13 to ≤15 g/dL (n=218)] and then randomly assigned to receive 300 U/kg PROCRIT, 100 U/kg PROCRIT or placebo by subcutaneous injection for 10 days before surgery, on the day of surgery, and for four days after surgery[14]. All patients received oral iron and a low dose postoperative warfarin regimen.[14]

Treatment with PROCRIT 300 U/kg significantly (p=0.024) reduced the risk of allogeneic transfusion in patients with a pretreatment hemoglobin of >10 to ≤13 g/dL; 5/31 (16%) of PROCRIT 300 U/kg, 6/26 (23%) of PROCRIT 100 U/kg and 13/29 (45%) of placebo-treated patients were transfused.[14] There was no significant difference in the number of patients transfused between PROCRIT (9% 300 U/kg, 6% 100 U/kg) and placebo (13%) in the >13 to ≤15 g/dL hemoglobin stratum. There were too few patients in the ≤10 g/dL group to determine if PROCRIT is useful in this hemoglobin strata.

In the >10 to ≤13 g/dL pretreatment stratum, the mean number of units transfused per PROCRIT-treated patient (0.45 units blood for 300 U/kg, 0.42 units blood for 100 U/kg) was less than the mean transfused per placebo-treated patient (1.14 units) (overall p=0.028). In addition, mean hemoglobin, hematocrit and reticulocyte counts increased significantly during the presurgery period in PROCRIT-treated patients.[14]

PROCRIT was also studied in an open-label, parallel-group trial enrolling 145 subjects with a pretreatment hemoglobin level of ≥10 to ≤13 g/dL who were scheduled for major orthopedic hip or knee surgery and who were not participating in an autologous program.[15] Subjects were randomly assigned to receive one of two subcutaneous dosing regimens of PROCRIT (600 U/kg once weekly for three weeks prior to surgery and on the day of surgery or 300 U/kg once daily for 10 days prior to surgery, on the day of surgery and for four days after surgery). All subjects received oral iron and appropriate pharmacologic anticoagulation therapy.

From pretreatment to presurgery, the mean increase in hemoglobin in 600 U/kg weekly group (1.44 g/dL) was greater than observed in the 300 U/kg daily group.[15] The mean increase in absolute reticulocyte count was smaller in the weekly group ($0.11 \times 10^6/mm^3$) compared to the daily group ($0.17 \times 10^6/mm^3$). Mean hemoglobin levels were similar for the two treatment groups throughout the postsurgical period.

The erythropoietic response observed in both treatment groups resulted in similar transfusion rates [11/69 (16%) in the 600 U/kg weekly group and 14/71 (20%) in the 300 U/kg daily group].[15] The mean number of units transfused per subject was approximately 0.3 units in both treatment groups.

CONTRAINDICATIONS

PROCRIT is contraindicated in patients with:

1) Uncontrolled hypertension.
2) Known hypersensitivity to mammalian cell-derived products.
3) Known hypersensitivity to Albumin (Human).

WARNINGS

Pediatric Use:

The multidose preserved formulation contains benzyl alcohol. Benzyl alcohol has been reported to be associated with an increased incidence of neurological and other complications in premature infants which are sometimes fatal. The safety and effectiveness of Epoetin alfa in children have not been established.

Thrombotic Events and Increased Mortality

A randomized, prospective trial of 1265 hemodialysis patients with clinically evident cardiac disease (ischemic heart disease or congestive heart failure) was conducted in which patients were assigned to PROCRIT treatment targeted to a maintenance hematocrit of either 42 ± 3% or 30 ± 3%. Increased mortality was observed in 634 patients randomized to a target hematocrit of 42% [221 deaths (35% mortality)] compared to 631 patients targeted to remain at a hematocrit of 30% [185 deaths (29% mortality)]. The reason for increased mortality observed in these studies is unknown, however the incidence of non-fatal myocardial infarctions (3.1% vs. 2.3%), vascular access thrombosis (39% vs. 29%) and all other thrombotic events (22% vs. 18%) were also higher in the group randomized to achieve a hematocrit of 42%.

Increased mortality was observed in a randomized placebo-controlled study of PROCRIT in patients who did not have chronic renal failure who were undergoing coronary artery bypass surgery (7 deaths in 126 patients randomized to PROCRIT vs. no deaths among 56 patients receiving placebo). Four of these deaths occurred during the period of study drug administration and all 4 deaths were associated with thrombotic events. While the extent of the population affected is unknown, in patients at risk for thrombosis, the anticipated benefits of PROCRIT treatment should be weighed against the potential for increased risks associated with therapy.

Chronic Renal Failure Patients

Hypertension: Patients with uncontrolled hypertension should not be treated with PROCRIT; blood pressure should be controlled adequately before initiation of therapy. Up to 80% of patients with CRF have a history of hypertension.[25] Although there does not appear to be any direct pressor effects of PROCRIT, blood pressure may rise during PROCRIT therapy. During the early phase of treatment when the hematocrit is increasing, approximately 25% of patients on dialysis may require initiation of, or increases in, antihypertensive therapy. Hypertensive encephalopathy and seizures have been observed in patients with CRF treated with PROCRIT.

Special care should be taken to closely monitor and aggressively control blood pressure in patients treated with PROCRIT. Patients should be advised as to the importance of compliance with antihypertensive therapy and dietary restrictions. If blood pressure is difficult to control by initiation of appropriate measures, the hematocrit may be reduced by decreasing or withholding the dose of PROCRIT. A clinically significant decrease in hematocrit may not be observed for several weeks.

It is recommended that the dose of PROCRIT be decreased if the hematocrit increase exceeds 4 points in any two-week period, because of the possible association of excessive rate of rise of hematocrit with an exacerbation of hypertension. In chronic renal failure patients on hemodialysis with clinically evident ischemic heart disease or congestive heart failure, the hematocrit should be managed carefully, not to exceed 36%. (see "Thrombotic Events")

Seizures: Seizures have occurred in patients with CRF participating in PROCRIT clinical trials.

In patients on dialysis, there was a higher incidence of seizures during the first 90 days of therapy (occurring in approximately 2.5% of patients) as compared with later timepoints.

Given the potential for an increased risk of seizures during the first 90 days of therapy, blood pressure and the presence of premonitory neurologic symptoms should be monitored closely. Patients should be cautioned to avoid potentially hazardous activities such as driving or operating heavy machinery during this period.

While the relationship between seizures and the rate of rise of hematocrit is uncertain, it is recommended that the dose of PROCRIT be decreased if the hematocrit increase exceeds 4 points in any two-week period.

Thrombotic Events: During hemodialysis, patients treated with PROCRIT may require increased anticoagulation with heparin to prevent clotting of the artificial kidney. (See "ADVERSE REACTIONS" for more information about thrombotic events.)

Other thrombotic events (e.g., myocardial infarction, cerebrovascular accident, transient ischemic attack) have occurred in clinical trials at an annualized rate of less than 0.04 events per patient-year of PROCRIT therapy. These trials were conducted in patients with CRF (whether on dialysis or not) in whom the target hematocrit was 32-40%. However, the risk of thrombotic events, including vascular access thromboses, was significantly increased in patients with ischemic heart disease or congestive heart failure receiving PROCRIT therapy with the goal of reaching a normal hematocrit (42%) as compared to a target hematocrit of 30%. Patients with pre-existing cardiovascular disease should be monitored closely.

Zidovudine-treated HIV-infected Patients

In contrast to CRF patients, PROCRIT therapy has not been linked to exacerbation of hypertension, seizures, and thrombotic events in HIV-infected patients.

PRECAUTIONS

The parenteral administration of any biologic product should be attended by appropriate precautions in case aller-

gic or other untoward reactions occur (see "CONTRAINDICATIONS"). In clinical trials, while transient rashes were occasionally observed concurrently with PROCRIT therapy, no serious allergic or anaphylactic reactions were reported. See "ADVERSE REACTIONS" for more information regarding allergic reactions.

The safety and efficacy of PROCRIT therapy have not been established in patients with a known history of a seizure disorder or underlying hematologic disease (e.g., sickle cell anemia, myelodysplastic syndromes, or hypercoagulable disorders).

In some female patients, menses have resumed following PROCRIT therapy; the possibility of pregnancy should be discussed and the need for contraception evaluated.

Hematology: Exacerbation of porphyria has been observed rarely in patients with CRF treated with PROCRIT. However, PROCRIT has not caused increased urinary excretion of porphyrin metabolites in normal volunteers, even in the presence of a rapid erythropoietic response. Nevertheless, PROCRIT should be used with caution in patients with known porphyria.

In preclinical studies in dogs and rats, but not in monkeys, PROCRIT therapy was associated with subclinical bone marrow fibrosis. Bone marrow fibrosis is a known complication of CRF in humans and may be related to secondary hyperparathyroidism or unknown factors. The incidence of bone marrow fibrosis was not increased in a study of patients on dialysis who were treated with PROCRIT for 12-19 months, compared to the incidence of bone marrow fibrosis in a matched group of patients who had not been treated with PROCRIT.

Hematocrit in CRF patients should be measured twice a week; zidovudine-treated HIV-infected and cancer patients should have hematocrit measured once a week until hematocrit has been stabilized, and measured periodically thereafter.

Delayed or Diminished Response: If the patient fails to respond or to maintain a response to doses within the recommended dosing range, the following etiologies should be considered and evaluated:

1) Iron deficiency: Virtually all patients will eventually require supplemental iron therapy. (See "Iron Evaluation").
2) Underlying infectious, inflammatory, or malignant processes.
3) Occult blood loss.
4) Underlying hematologic diseases (i.e., thalassemia, refractory anemia, or other myelodysplastic disorders).
5) Vitamin deficiencies: folic acid or vitamin B12.
6) Hemolysis.
7) Aluminum intoxication.
8) Osteitis fibrosa cystica.

Iron Evaluation: During PROCRIT therapy, absolute or functional iron deficiency may develop. Functional iron deficiency, with normal ferritin levels but low transferrin saturation, is presumably due to the inability to mobilize iron stores rapidly enough to support increased erythropoiesis. Transferrin saturation should be at least 20% and ferritin should be at least 100 ng/mL.

Prior to and during PROCRIT therapy, the patient's iron status, including transferrin saturation (serum iron divided by iron binding capacity) and serum ferritin, should be evaluated. Virtually all patients will eventually require supplemental iron to increase or maintain transferrin saturation to levels which will adequately support erythropoiesis stimulated by PROCRIT. All surgery patients being treated with PROCRIT should receive adequate iron supplementation throughout the course of therapy in order to support erythropoiesis and avoid depletion of iron stores.

Drug Interactions: No evidence of interaction of PROCRIT with other drugs was observed in the course of clinical trials.

Carcinogenesis, Mutagenesis, and Impairment of Fertility: Carcinogenic potential of PROCRIT has not been evaluated. PROCRIT does not induce bacterial gene mutation (Ames Test), chromosomal aberrations in mammalian cells, micronuclei in mice, or gene mutation at the HGPRT locus. In female rats treated intravenously with PROCRIT, there was a trend for slightly increased fetal wastage at doses of 100 and 500 Units/kg.

Pregnancy Category C: PROCRIT has been shown to have adverse effects in rats when given in doses five times the human dose. There are no adequate and well-controlled studies in pregnant women. PROCRIT should be used during pregnancy only if potential benefit justifies the potential risk to the fetus.

In studies in female rats, there were decreases in body weight gain, delays in appearance of abdominal hair, delayed eyelid opening, delayed ossification, and decreases in the number of caudal vertebrae in the F1 fetuses of the 500 Units/kg group. In female rats treated intravenously, there was a trend for slightly increased fetal wastage at doses of 100 and 500 Units/kg. PROCRIT has not shown any adverse effect at doses as high as 500 Units/kg in pregnant rabbits (from day 6 to 18 of gestation).

Nursing Mothers: Postnatal observations of the live offspring (F1 generation) of female rats treated with PROCRIT during gestation and lactation revealed no effect of PROCRIT at doses of up to 500 Units/kg. There were, however, decreases in body weight gain, delays in appearance of abdominal hair, eyelid opening, and decreases in the number of caudal vertebrae in the F1 fetuses of the 500 Units/kg group. There were no effects related to PROCRIT on the F2 generation fetuses.

It is not known whether PROCRIT is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when PROCRIT is administered to a nursing woman.

Pediatric Use: The safety and effectiveness of PROCRIT in children have not been established (See "WARNINGS").

Chronic Renal Failure Patients

Patients with CRF Not Requiring Dialysis: Blood pressure and hematocrit should be monitored no less frequently than for patients maintained on dialysis. Renal function and fluid and electrolyte balance should be closely monitored, as an improved sense of well-being may obscure the need to initiate dialysis in some patients.

Hematology: Sufficient time should be allowed to determine a patient's responsiveness to a dosage of PROCRIT before adjusting the dose. Because of the time required for erythropoiesis and the red cell half-life, an interval of 2-6 weeks may occur between the time of a dose adjustment (initiation, increase, decrease, or discontinuation) and a significant change in hematocrit.

In order to avoid reaching the suggested target hematocrit too rapidly, or exceeding the suggested target range (hematocrit of 30-36%), the guidelines for dose and frequency of dose adjustments (see "DOSAGE AND ADMINISTRATION") should be followed.

For patients who respond to PROCRIT with a rapid increase in hematocrit (e.g., more than 4 points in any two-week period), the dose of PROCRIT should be reduced because of the possible association of excessive rate of rise of hematocrit with an exacerbation of hypertension.

The elevated bleeding time characteristic of CRF decreases toward normal after correction of anemia in patients treated with PROCRIT. Reduction of bleeding time also occurs after correction of anemia by transfusion.

Laboratory Monitoring: The hematocrit should be determined twice a week until it has stabilized in the suggested target range and the maintenance dose has been established. After any dose adjustment, the hematocrit should also be determined twice weekly for at least 2-6 weeks until it has been determined that the hematocrit has stabilized in response to the dose change. The hematocrit should then be monitored at regular intervals.

A complete blood count with differential and platelet count should be performed regularly. During clinical trials, modest increases were seen in platelets and white blood cell counts. While these changes were statistically significant, they were not clinically significant and the values remained within normal ranges.

In patients with CRF, serum chemistry values [including blood urea nitrogen (BUN), uric acid, creatinine, phosphorus, and potassium] should be monitored regularly. During clinical trials in patients on dialysis, modest increases were seen in BUN, creatinine, phosphorus, and potassium. In some patients with CRF not on dialysis, treated with PROCRIT, modest increases in serum uric acid and phosphorus were observed. While changes were statistically significant, the values remained within the ranges normally seen in patients with CRF.

Diet: As the hematocrit increases and patients experience an improved sense of well-being and quality of life, the importance of compliance with dietary and dialysis prescriptions should be reinforced. In particular, hyperkalemia is not uncommon in patients with CRF. In U.S. studies in patients on dialysis, hyperkalemia has occurred at an annualized rate of approximately 0.11 episodes per patient-year of PROCRIT therapy, often in association with poor compliance to medication, diet and/or dialysis.

Dialysis Management: Therapy with PROCRIT results in an increase in hematocrit and a decrease in plasma volume which could affect dialysis efficiency. In studies to date, the resulting increase in hematocrit did not appear to adversely affect dialyzer function[9,10] or the efficiency of high flux hemodialysis.[11] During hemodialysis, patients treated with PROCRIT may require increased anticoagulation with heparin to prevent clotting of the artificial kidney.

Patients who are marginally dialyzed may require adjustments in their dialysis prescription. As with all patients on dialysis, the serum chemistry values (including BUN, creatinine, phosphorus, and potassium) in patients treated with PROCRIT should be monitored regularly to assure the adequacy of the dialysis prescription.

Information for Patients: In those situations in which the physician determines that a home dialysis patient can safely and effectively self-administer PROCRIT, the patient should be instructed as to the proper dosage and administration. Home dialysis patients should be referred to the full "INFORMATION FOR HOME DIALYSIS PATIENTS" section attached; it is not a disclosure of all possible effects. Patients should be informed of the signs and symptoms of allergic drug reaction and advised of appropriate actions. If home use is prescribed for a home dialysis patient, the patient should be thoroughly instructed in the importance of proper disposal and cautioned against the reuse of needles, syringes, or drug product. A puncture-resistant container for the disposal of used syringes and needles should be available to the patient. The full container should be disposed of according to the directions provided by the physician.

Renal Function: In patients with CRF not on dialysis, renal function and fluid and electrolyte balance should be closely monitored, as an improved sense of well-being may obscure the need to initiate dialysis in some patients. In patients with CRF not on dialysis, placebo-controlled studies of progression of renal dysfunction over periods of greater than one year have not been completed. In shorter-term trials in patients with CRF not on dialysis, changes in creatinine and creatinine clearance were not significantly different in patients treated with PROCRIT, compared with placebo-treated patients. Analysis of the slope of 1/serum creatinine vs. time plots in these patients indicates no significant change in the slope after the initiation of PROCRIT therapy.

Zidovudine-treated HIV-infected Patients

Hypertension: Exacerbation of hypertension has not been observed in zidovudine-treated HIV-infected patients treated with PROCRIT. However, PROCRIT should be withheld in these patients if pre-existing hypertension is uncontrolled, and should not be started until blood pressure is controlled. In double-blind studies, a single seizure has been experienced by a patient treated with PROCRIT.[22]

Cancer Patients on Chemotherapy

Hypertension: Hypertension, associated with a significant increase in hematocrit, has been noted rarely in cancer patients treated with PROCRIT. Nevertheless, blood pressure in patients treated with PROCRIT should be monitored carefully, particularly in patients with an underlying history of hypertension or cardiovascular disease.

Seizures: In double-blind, placebo-controlled trials, 3.2% (N=2/63) of patients treated with PROCRIT and 2.9% (N=2/68) of placebo-treated patients had seizures. Seizures in 1.6% (N=1/63) of patients treated with PROCRIT occurred in the context of a significant increase in blood pressure and hematocrit from baseline values. However, both patients treated with PROCRIT also had underlying CNS pathology which may have been related to seizure activity.

Thrombotic Events: In double-blind, placebo-controlled trials, 3.2% (N=2/63) of patients treated with PROCRIT and 11.8% (N=8/68) of placebo-treated patients had thrombotic events (e.g. pulmonary embolism, cerebrovascular accident).

Growth Factor Potential: PROCRIT is a growth factor that primarily stimulates red cell production. However, the possibility that PROCRIT can act as a growth factor for any tumor type, particularly myeloid malignancies, cannot be excluded.

Surgery Patients

Thrombotic/Vascular Events: In perioperative clinical trials with orthopedic patients, the overall incidence of thrombotic/vascular events was similar in Epoetin alfa and placebo-treated patients who had a pretreatment hemoglobin of >10 to ≤13 g/dL. In patients with a hemoglobin of >13 g/dL treated with 300 U/kg of Epoetin alfa, the possibility that PROCRIT treatment may be associated with an increased risk of postoperative thrombotic/vascular events cannot be excluded.[14-16,24]

In one study in which Epoetin alfa was administered in the perioperative period to patients undergoing coronary artery bypass graft surgery, there were seven deaths in the Epoetin alfa-treated groups (N=126) and no deaths in the placebo-treated group (N=56). Among the seven deaths in the Epoetin alfa-treated patients, four were at the time of therapy (between study day 2 and 8). The four deaths at the time of therapy (3%) were associated with thrombotic/vascular events. A causative role of Epoetin alfa cannot be excluded. (See "WARNINGS")

Hypertension: Blood pressure may rise in the perioperative period in patients being treated with PROCRIT. Therefore, blood pressure should be monitored carefully.

ADVERSE REACTIONS

Chronic Renal Failure Patients

Studies analyzed to date indicate that PROCRIT is generally well-tolerated. The adverse events reported are frequent sequelae of CRF and are not necessarily attributable to PROCRIT therapy. In double-blind, placebo-controlled studies involving over 300 patients with CRF, the events reported in greater than 5% of patients treated with PROCRIT during the blinded phase were:

PERCENT OF PATIENTS REPORTING EVENT

Event	Patients Treated with epoetin alfa (N=200)	PLACEBO-Treated Patients (N=135)
Hypertension	24%	19%
Headache	16%	12%
Arthralgias	11%	6%
Nausea	11%	9%
Edema	9%	10%
Fatigue	9%	14%
Diarrhea	9%	6%
Vomiting	8%	5%
Chest Pain	7%	9%
Skin Reaction (Administration Site)	7%	12%
Asthenia	7%	12%
Dizziness	7%	13%
Clotted Access	7%	2%

Significant adverse events of concern in patients with CRF treated in double-blinded, placebo-controlled trials occurred

Continued on next page

Procrit—Cont.

in the following percent of patients during the blinded phase of the studies:

Seizure	1.1%	1.1%
CVA / TIA	0.4%	0.6%
MI	0.4%	1.1%
Death	0	1.7%

In the U.S. PROCRIT studies in patients on dialysis (over 567 patients), the incidence (number of events per patient-year) of the most frequently reported adverse events were: hypertension (0.75), headache (0.40), tachycardia (0.31), nausea/vomiting (0.26), clotted vascular access (0.25), shortness of breath (0.14), hyperkalemia (0.11), and diarrhea (0.11). Other reported events occurred at a rate of less than 0.10 events per patient per year.

Events reported to have occurred within several hours of administration of PROCRIT were rare, mild, and transient, and included injection site stinging in dialysis patients and flu-like symptoms such as arthralgias and myalgias.

In all studies analyzed to date, PROCRIT administration was generally well-tolerated, irrespective of the route of administration.

Hypertension: Increases in blood pressure have been reported in clinical trials, often during the first 90 days of therapy. On occasion, hypertensive encephalopathy and seizures have been observed in patients with CRF treated with PROCRIT. When data from all patients in the U.S. Phase III multicenter trial were analyzed, there was an apparent trend of more reports of hypertensive adverse events in patients on dialysis with a faster rate of rise of hematocrit (greater than 4 hematocrit points in any two-week period). However, in a double-blind, placebo-controlled trial, hypertensive adverse events were not reported at an increased rate in the group treated with PROCRIT (150 Units/kg T.I.W.) relative to the placebo group.

Seizures: There have been 47 seizures in 1,010 patients on dialysis treated with PROCRIT in clinical trials, with an exposure of 986 patient-years for a rate of approximately 0.048 events per patient-year. However, there appeared to be a higher rate of seizures during the first 90 days of therapy (occurring in approximately 2.5% of patients) when compared to subsequent 90-day periods. The baseline incidence of seizures in the untreated dialysis population is difficult to determine; it appears to be in the range of 5-10% per patient-year.[26-28]

Thrombotic Events: In clinical trials where the maintenance hematocrit was 35 ± 3% on PROCRIT, clotting of the vascular access (A-V shunt) has occurred at an annualized rate of about 0.25 events per patient-year, and other thrombotic events (myocardial infarction, cerebrovascular accident, transient ischemic attack, and pulmonary embolism) occurred at a rate of 0.04 events per patient-year. In a separate study of 1,111 untreated dialysis patients, clotting of the vascular access occurred at a rate of 0.5 events per patient-year. However, in chronic renal failure patients on hemodialysis who also had clinically evident ischemic heart disease or congestive heart failure, the risk of A-V shunt thrombosis was higher (39% vs 29%, p<0.001), and myocardial infarction, vascular ischemic events, and venous thrombosis were increased in patients targeted to a hematocrit of 42 ± 3% compared to those maintained at 30 ± 3%. (see "WARNINGS")

In patients treated with commercial PROCRIT, there have been rare reports of serious or unusual thrombo-embolic events including migratory thrombophlebitis, microvascular thrombosis, pulmonary embolus, and thrombosis of the retinal artery, and temporal and renal veins. A causal relationship has not been established.

Allergic Reactions: There have been no reports of serious allergic reactions or anaphylaxis associated with PROCRIT administration during clinical trials. Skin rashes and urticaria have been observed rarely and when reported have generally been mild and transient in nature.

In over 125,000 patients treated with commercial PROCRIT, there have been rare reports of potentially serious allergic reactions including urticaria with associated respiratory symptoms or circumoral edema (<0.0001 events per patient-year), or urticaria alone (<0.0001 events per patient-year). Most reactions occurred in situations where a casual relationship could not be established. Many of these patients resumed PROCRIT therapy without recurrence of symptoms, some in conjunction with antihistamine pretreatment. However, symptoms recurred with rechallenge in a few instances, suggesting that allergic reactivity, although rare, may occasionally be associated with PROCRIT therapy.

There has been no evidence for development of antibodies to erythropoietin in patients tested to date, including those receiving PROCRIT for over 4 years. Nevertheless, if an anaphylactoid reaction occurs, PROCRIT should be immediately discontinued and appropriate therapy initiated.

Zidovudine-treated HIV-infected Patients

Adverse events reported in clinical trials with PROCRIT in zidovudine-treated HIV-infected patients were consistent with the progression of HIV infection. In double-blind, placebo-controlled studies of three-months duration involving approximately 300 zidovudine-treated HIV-infected patients, adverse events with an incidence of ≥10% in either patients treated with PROCRIT or placebo-treated patients were:

Percent of Patients Reporting Event

Event	Patients Treated with PROCRIT (N=144)	PLACEBO-Treated Patients (N=153)
Pyrexia	38%	29%
Fatigue	25%	31%
Headache	19%	14%
Cough	18%	14%
Diarrhea	16%	18%
Rash	16%	8%
Congestion, Respiratory	15%	10%
Nausea	15%	12%
Shortness of Breath	14%	13%
Asthenia	11%	14%
Skin Reaction, (Administration Site)	10%	7%
Dizziness	9%	10%

There were no statistically significant differences between treatment groups in the incidence of the above events.

In the 297 patients studied, PROCRIT was not associated with significant increases in opportunistic infections or mortality.[22] In 71 patients from this group treated with PROCRIT at 150 Units/kg (T.I.W.), serum p24 antigen levels did not appear to increase.[23] Preliminary data showed no enhancement of HIV replication in infected cell lines *in vitro*.[22]

Peripheral white blood cell and platelet counts are unchanged following PROCRIT therapy.

Allergic Reactions: Two zidovudine-treated HIV-infected patients had urticarial reactions within 48 hours of their first exposure to study medication. One patient was treated with PROCRIT and one was treated with placebo (PROCRIT vehicle alone). Both patients had positive immediate skin tests against their study medication with a negative saline control. The basis for this apparent pre-existing hypersensitivity to components of the PROCRIT formulation is unknown, but may be related to HIV-induced immunosuppression or prior exposure to blood products.

Seizures: In double-blind and open-label trials of PROCRIT in zidovudine-treated HIV-infected patients, ten patients have experienced seizures.[22] In general, these seizures appear to be related to underlying pathology such as meningitis or cerebral neoplasms, not PROCRIT therapy.

Cancer Patients on Chemotherapy

Adverse experiences reported in clinical trials with PROCRIT in cancer patients were consistent with the underlying disease state. In double-blind, placebo-controlled studies of up to 3-months duration involving 131 cancer patients, adverse events with an incidence >10% in either patients treated with PROCRIT or placebo-treated patients were as indicated below.

Percent of Patients Reporting Event

Event	Patients Treated with PROCRIT (N=63)	PLACEBO-Treated Patients (N=68)
Pyrexia	29%	19%
Diarrhea	21%[a]	7%
Nausea	17%[b]	32%
Vomiting	17%	15%
Edema	17%[c]	1%
Asthenia	13%	16%
Fatigue	13%	15%
Shortness of Breath	13%	9%
Paresthesia	11%	6%
Upper Respiratory Infection	11%	4%
Dizziness	5%	12%
Trunk Pain	3%[d]	16%

[a] p = 0.041 [c] p = 0.0016
[b] p = 0.069 [d] p = 0.017

Although some statistically significant differences between patients treated with PROCRIT and placebo-treated patients were noted, the overall safety profile of PROCRIT appeared to be consistent with the disease process of advanced cancer. During double-blind and subsequent open-label therapy in which patients (N=72 for total exposure to PROCRIT) were treated for up to 32 weeks with doses as high as 927 Units/kg, the adverse experience profile of PROCRIT was consistent with the progression of advanced cancer.

Based on comparable survival data and on the percentage of patients treated with PROCRIT and placebo-treated patients who discontinued therapy due to death, disease progression or adverse experiences (22% and 13%, respectively; p = 0.25), the clinical outcome in patients treated with PROCRIT and placebo-treated patients appeared to be similar. Available data from animal tumor models and measurement of proliferation of solid tumor cells from clinical biopsy specimens in response to PROCRIT suggest that PROCRIT does not potentiate tumor growth. Nevertheless, as a growth factor, the possibility that PROCRIT may potentiate growth of some tumors, particularly myeloid tumors, cannot be excluded. A randomized controlled Phase IV study is currently ongoing to further evaluate this issue.

The mean peripheral white blood cell count was unchanged following PROCRIT therapy compared to the corresponding value in the placebo-treated group.

Surgery Patients

Adverse events with an incidence of ≥10% are shown in the following table:

[See table below]

Thrombotic/vascular events: In three double-blind, placebo-controlled orthopedic surgery studies, the rate of deep venous thrombosis (DVT) was similar among Epoetin alfa and placebo-treated patients in the recommended population of patients with a pretreatment hemoglobin of >10 to ≤13 g/dL.[14,16,24] However, in 2 of 3 orthopedic surgery studies the overall rate (all pretreatment hemoglobin groups combined) of DVTs detected by postoperative ultrasonography and/or surveillance venography was higher in the Epoetin alfa-treated group than in the placebo-treated group (11% vs. 6%). This finding was attributable to the difference in DVT rates observed in the subgroup of patients with pretreatment hemoglobin >13 g/dL. However, the incidence of DVTs was within the range of that reported in the literature for orthopedic surgery patients.

In the orthopedic surgery study of patients with pretreatment hemoglobin of >10 to ≤13 g/dL which compared two dosing regimens (600 U/kg weekly × 4 and 300 U/kg daily × 15), four subjects in the 600 U/kg weekly PROCRIT group (5%) and no subjects in the 300 U/kg daily group had a thrombotic vascular event during the study period.[15]

In a study examining the use of Epoetin alfa in 182 patients scheduled for coronary artery bypass graft surgery, 23% of patients treated with Epoetin alfa and 29% treated with placebo experienced thrombotic/vascular events. There were 4 deaths among the Epoetin alfa-treated patients that were associated with a thrombotic/vascular event. A causative role of Epoetin alfa cannot be excluded. (See "WARNINGS")

Percent of Patients Reporting Event

Event	Patients Treated with PROCRIT 300 U/kg (N=112)[a]	Patients Treated with PROCRIT 100 U/kg (N=101)[b]	PLACEBO-Treated Patients (N=103)[a]	PROCRIT 600 U/kg (N=73)[b]	PROCRIT 300 U/kg (N=72)[b]
Pyrexia	51%	50%	60%	47%	42%
Nausea	48%	43%	45%	45%	58%
Constipation	43%	42%	43%	51%	53%
Skin Reaction, (Administration Site)	25%	19%	22%	26%	29%
Vomiting	22%	12%	14%	21%	29%
Skin Pain	18%	18%	17%	5%	4%
Pruritus	16%	16%	14%	14%	22%
Insomnia	13%	16%	13%	21%	15%
Headache	13%	11%	9%	10%	19%
Dizziness	12%	9%	12%	11%	21%
Urinary Tract Infection	12%	3%	11%	11%	8%
Hypertension	10%	11%	10%	5%	10%
Diarrhea	10%	7%	12%	10%	6%
Deep Venous Thrombosis	10%	3%	5%	0%[c]	0%[c]
Dyspepsia	9%	11%	6%	7%	8%
Anxiety	7%	2%	11%	11%	4%
Edema	6%	11%	8%	11%	7%

[a] Study including patients undergoing orthopedic surgery treated with PROCRIT or placebo for 15 days
[b] Study including patients undergoing orthopedic surgery treated with PROCRIT 600 U/kg weekly × 4 or 300 U/kg daily × 15
[c] Determined by clinical symptoms

OVERDOSAGE

The maximum amount of PROCRIT that can be safely administered in single or multiple doses has not been determined. Doses of up to 1,500 Units/kg (T.I.W.) for three to four weeks have been administered without any direct toxic effects of PROCRIT itself.[6] Therapy with PROCRIT can result in polycythemia if the hematocrit is not carefully mon-

ferred and the dose appropriately adjusted. If the suggested target range is exceeded, PROCRIT may be temporarily withheld until the hematocrit returns to the suggested target range; PROCRIT therapy may then be resumed using a lower dose (see "DOSAGE AND ADMINISTRATION"). If polycythemia is of concern, phlebotomy may be indicated to decrease the hematocrit.

DOSAGE AND ADMINISTRATION

Chronic Renal Failure Patients

Starting doses of PROCRIT over the range of 50-100 Units/kg three times weekly (T.I.W.) have been shown to be safe and effective in increasing hematocrit and eliminating transfusion dependency in patients with CRF (see "Clinical Experience"). The dose of PROCRIT should be reduced as the hematocrit approaches 36% or increases by more than 4 points in any 2-week period. The dosage of PROCRIT must be individualized to maintain the hematocrit within the suggested target range. At the physician's discretion, the suggested target hematocrit range may be expanded to achieve maximal patient benefit.

PROCRIT may be given either as an intravenous (IV) or subcutaneous (SC) injection. In patients on hemodialysis, PROCRIT usually has been administered as an IV bolus (T.I.W.). While the administration of PROCRIT is independent of the dialysis procedure, PROCRIT may be administered into the venous line at the end of the dialysis procedure to obviate the need for additional venous access. In patients with CRF not on dialysis, PROCRIT may be given either as an IV or SC injection.

Home hemodialysis patients who have been judged competent by their physicians to self-administer PROCRIT without medical or other supervision may give themselves either an IV or SC injection. The table below provides general therapeutic guidelines for patients with CRF:

[See table above]

During therapy, hematological parameters should be monitored regularly (see "Laboratory Monitoring").

Pre-Therapy Iron Evaluation: Prior to and during PROCRIT therapy, the patient's iron stores, including transferrin saturation (serum iron divided by iron binding capacity) and serum ferritin, should be evaluated. Transferrin saturation should be at least 20%, and ferritin should be at least 100 ng/mL. Virtually all patients will eventually require supplemental iron to increase or maintain transferrin saturation to levels that will adequately support erythropoiesis stimulated by PROCRIT.

Dose Adjustment: Following PROCRIT therapy, a period of time is required for erythroid progenitors to mature and be released into circulation resulting in an eventual increase in hematocrit. Additionally, red blood cell survival time affects hematocrit and may vary due to uremia. As a result, the time required to elicit a clinically significant change in hematocrit (increase or decrease) following any dose adjustment may be 2-6 weeks.

Dose adjustment should not be made more frequently than once a month, unless clinically indicated. After any dose adjustment, the hematocrit should be determined twice weekly for at least 2-6 weeks (see "Laboratory Monitoring").

- If the hematocrit is increasing and approaching 36%, the dose should be reduced to maintain the suggested target hematocrit range. If the reduced dose does not stop the rise in hematocrit, and it exceeds 36%, doses should be temporarily withheld until the hematocrit begins to decrease, at which point therapy should be reinitiated at a lower dose.
- At any time, if the hematocrit increases by more than 4 points in a 2-week period, the dose should be immediately decreased. After the dose reduction, the hematocrit should be monitored twice weekly for 2-6 weeks, and further dose adjustments should be made as outlined in "Maintenance Dose".
- If a hematocrit increase of 5-6 points is not achieved after an 8-week period and iron stores are adequate (see "Delayed or Diminished Response"), the dose of PROCRIT may be incrementally increased. Further increases may be made at 4-6 week intervals until the desired response is attained.

Maintenance Dose: The maintenance dose must be individualized for each patient on dialysis. In the U.S. Phase III multicenter trial in patients on hemodialysis, the median maintenance dose was 75 Units/kg (T.I.W.), with a range from 12.5 to 525 Units/kg (T.I.W.). Almost 10% of the patients required a dose of 25 Units/kg, or less, and approximately 10% of the patients required more than 200 Units/kg (T.I.W.) to maintain their hematocrit in the suggested target range.

If the hematocrit remains below, or falls below, the suggested target range, iron stores should be re-evaluated. If the transferrin saturation is less than 20%, supplemental iron should be administered. If the transferrin saturation is greater than 20%, the dose of PROCRIT may be increased. Such dose increases should not be made more frequently than once a month, unless clinically indicated, as the response time of the hematocrit to a dose increase can be 2-6 weeks. Hematocrit should be measured twice weekly for 2-6 weeks following dose increases. In patients with CRF not on dialysis, the maintenance dose must also be individualized. PROCRIT doses of 75-150 Units/kg per week have been shown to maintain hematocrits of 36-38% for up to 6 months.

Delayed or Diminished Response: Over 95% of patients with CRF responded with clinically significant increases in hematocrit, and virtually all patients were transfusion-independent within approximately two months of initiation of PROCRIT therapy.

If a patient fails to respond or maintain a response, other etiologies should be considered and evaluated as clinically indicated. (See "PRECAUTIONS" section for discussion of delayed or diminished response.)

Starting Dose	Reduce Dose If	Increase Dose When	Maintenance Dose	Suggested Hct. Range
50-100 Units/kg T.I.W.; IV or SC	1) Hct. approaches 36%, or 2) Hct. increases > 4 points in any 2-week period	Hct. does not increase by 5-6 points after 8 weeks of therapy, and hct. is below suggested target range	Individually titrate	30-36%

Zidovudine-treated HIV-infected Patients

Prior to beginning PROCRIT, it is recommended that the endogenous serum erythropoietin level be determined (prior to transfusion). Available evidence suggests that patients receiving zidovudine with endogenous serum erythropoietin levels > 500 mUnits/mL are unlikely to respond to therapy with PROCRIT.

Starting Dose: For patients with serum erythropoietin levels ≤ 500 mUnits/mL who are receiving a dose of zidovudine ≤ 4,200 mg/week, the recommended starting dose of PROCRIT is 100 Units/kg as an intravenous or subcutaneous injection three times weekly (T.I.W.) for 8 weeks.

Increase Dose: During the dose adjustment phase of therapy, the hematocrit should be monitored weekly. If the response is not satisfactory in terms of reducing transfusion requirements or increasing hematocrit after 8 weeks of therapy, the dose of PROCRIT can be increased by 50-100 Units/kg (T.I.W.). Response should be evaluated every 4-8 weeks thereafter and the dose adjusted accordingly by 50-100 Units/kg increments (T.I.W.). If patients have not responded satisfactorily to a PROCRIT dose of 300 Units/kg (T.I.W.), it is unlikely that they will respond to higher doses of PROCRIT.

Maintenance Dose: After attainment of the desired response (i.e., reduced transfusion requirements or increased hematocrit), the dose of PROCRIT should be titrated to maintain the response based on factors such as variations in zidovudine dose and the presence of intercurrent infectious or inflammatory episodes. If the hematocrit exceeds 40%, the dose should be discontinued until the hematocrit drops to 36%. The dose should be reduced by 25% when treatment is resumed and then titrated to maintain the desired hematocrit.

Cancer Patients on Chemotherapy

Baseline endogenous serum erythropoietin levels varied among patients in these trials with approximately 75% (N=83/110) having endogenous serum erythropoietin levels < 132 mUnits/mL, and approximately 4% (N=4/110) of patients having endogenous serum erythropoietin levels > 500 mUnits/mL. In general, patients with lower baseline serum erythropoietin levels responded more vigorously to PROCRIT than patients with higher erythropoietin levels. Although no specific serum erythropoietin level can be stipulated above which patients would be unlikely to respond to PROCRIT therapy, treatment of patients with grossly elevated serum erythropoietin levels (e.g., > 200 mUnits/mL) is not recommended. The hematocrit should be monitored on a weekly basis in patients receiving PROCRIT therapy until hematocrit becomes stable.

Starting Dose: The recommended starting dose of PROCRIT is 150 Units/kg subcutaneously (T.I.W.).

Dose Adjustment: If the response is not satisfactory in terms of reducing transfusion requirements or increasing hematocrit after 8 weeks of therapy, the dose of PROCRIT can be increased up to 300 Units/kg (T.I.W.). If patients have not responded satisfactorily to a PROCRIT dose of 300 Units/kg (T.I.W.), it is unlikely that they will respond to higher doses of PROCRIT. If the hematocrit exceeds 40%, the dose of PROCRIT should be withheld until the hematocrit falls to 36%. The dose of PROCRIT should be reduced by 25% when treatment is resumed and titrated to maintain the desired hematocrit. If the initial dose of PROCRIT includes a very rapid hematocrit response (e.g., an increase of more than 4 percentage points in any 2-week period), the dose of PROCRIT should be reduced.

Surgery Patients

Prior to initiating treatment with PROCRIT a hemoglobin should be obtained to establish that it is >10 to ≤13 g/dL.[14] The recommended dose of PROCRIT is 300 U/kg/day subcutaneously for 10 days before surgery, on the day of surgery, and for 4 days after surgery.[14]

An alternate dose schedule is 600 U/kg PROCRIT subcutaneously in once weekly doses (21, 14 and 7 days before surgery) plus a fourth dose on day of surgery.[15]

All patients should receive adequate iron supplementation. Iron supplementation should be initiated no later than the beginning of treatment with PROCRIT and should continue throughout the course of therapy.

PREPARATION AND ADMINISTRATION OF PROCRIT

1. DO NOT SHAKE. It is not necessary to shake PROCRIT. Prolonged vigorous shaking may denature any glycoprotein, rendering it biologically inactive.
2. Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration. Do not use any vials exhibiting particulate matter or discoloration.
3. Using aseptic techniques, attach a sterile needle to a sterile syringe. Remove the flip top from the vial containing PROCRIT, and wipe the septum with a disinfectant. Insert the needle into the vial, and withdraw into the syringe an appropriate volume of solution.
4. **Single-dose** 1 mL vial contains no preservative. Use one dose per vial; do not re-enter vial. Discard unused portions. **Multidose** 1 mL and 2 mL vials contain preservative. Store at 2 to 8°C after initial entry and between doses. Discard 21 days after initial entry.
5. Do not dilute or administer in conjunction with other drug solutions. However, at the time of subcutaneous administration, preservative-free PROCRIT from single-use vials may be admixed in a syringe with bacteriostatic 0.9% sodium chloride injection, USP, with benzyl alcohol 0.9% (bacteriostatic saline) at a 1:1 ratio using aseptic technique. The benzyl alcohol in the bacteriostatic saline acts as a local anesthetic which may ameliorate subcutaneous injection site discomfort. Admixing is not necessary when using the multidose vials of PROCRIT containing benzyl alcohol.

HOW SUPPLIED

PROCRIT, containing Epoetin alfa, is available in vials containing color coded labels.

1 mL **Single-Dose, Preservative-Free** Solution

Each dosage form is supplied in the following packages:

Cartons containing six (6) **single-dose** vials:
- 2,000 Units/mL (NDC 59676-302-01) (Purple)
- 3,000 Units/mL (NDC 59676-303-01) (Magenta)
- 4,000 Units/mL (NDC 59676-304-01) (Green)
- 10,000 Units/mL (NDC 59676-310-01) (Red)

Cartons containing four (4) **single-dose** vials:
- 40,000 Units/mL (NDC 59676-340-01) (Orange)

Trays containing twenty-five (25) **single-dose** vials:
- 2,000 Units/mL (NDC 59676-302-02) (Purple)
- 3,000 Units/mL (NDC 59676-303-02) (Magenta)
- 4,000 Units/mL (NDC 59676-304-02) (Green)
- 10,000 Units/mL (NDC 59676-310-02) (Red)

2 mL **Multidose, Preserved** Solution

Cartons containing six (6) **multidose** vials:
- 10,000 Units/mL (NDC 59676-312-01) (Blue)

1 mL **Multidose, Preserved** Solution

Cartons containing six (6) **multidose** vials:
- 20,000 Units/mL (NDC 59676-320-01) (Lime)

STORAGE

Store at 2° to 8° C (36° to 46° F). Do not freeze or shake.

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PROCRIT®
EPOETIN ALFA

INFORMATION FOR HOME DIALYSIS PATIENTS

What is PROCRIT and how does it work?

PROCRIT is a copy of human erythropoietin, a hormone produced primarily by healthy kidneys. PROCRIT replaces the erythropoietin that the failed kidneys can no longer produce, and signals the bone marrow to make the oxygen-carrying red blood cells once again. PROCRIT is produced in mammalian cells that have been genetically altered by the addition of a gene of the natural substance erythropoietin.

How should I take PROCRIT?

In those situations where your doctor has determined that you, as a home dialysis patient, can self-administer PROCRIT, you will receive instruction on how much PROCRIT to use, how to inject it, how often you should inject it, and how you should dispose of the unused portions of each vial.

You will be instructed to monitor your blood pressure carefully everyday and to report any changes outside of the guidelines that your doctor has given you. When the number of red blood cells increases, your blood pressure can also increase, so your doctor may prescribe some new or additional blood pressure medication. Be sure to follow your doctor's orders. You may also be instructed to have certain laboratory tests, such as additional hematocrit or iron level measurements, done more frequently. You may be asked to report these tests to your doctor or dialysis center. Also, your doctor may prescribe additional iron for you to take. Be sure to comply with your doctor's orders.

Continue to check your access, as your doctor or nurse has shown you, to make sure it is working. Be sure to let your health care professional know right away if there is a problem.

Allergy to PROCRIT

Patients occasionally experience redness, swelling, or itching at the site of injection of PROCRIT. This may indicate an allergy to the components of PROCRIT, or it may indicate a local reaction. If you have a local reaction, consult your doctor. A potentially more serious reaction would be a generalized allergy to PROCRIT, which could cause a rash over the whole body, shortness of breath, wheezing, reduction in blood pressure, fast pulse, or sweating. Severe cases of generalized allergy may be life-threatening. If you think you are having a generalized allergic reaction, stop taking PROCRIT and notify a doctor or emergency medical personnel immediately.

How will I know if PROCRIT is working?

The effectiveness of PROCRIT is measured by the increase in hematocrit (the amount of red blood cells in the blood) that results from PROCRIT therapy. The rise in hematocrit is not immediate. It usually takes about 2-6 weeks before the hematocrit starts to rise. The amount of time it takes, and the dose of PROCRIT that is needed to make the hematocrit increase, varies from patient to patient.

What is the most important information I should know about PROCRIT and CHRONIC RENAL FAILURE?

PROCRIT has been prescribed for you by your doctor because you:

1. Have anemia due to your kidney disease.
2. Are able to dialyze at home.
3. Have been determined to be able to administer PROCRIT without direct medical or other supervision.

A lack of energy or feeling of tiredness is the major symptom of anemia. Additional symptoms include shortness of breath, chest pain, and feeling cold all the time. The reason for these symptoms is that there is a lack of red blood cells. Red blood cells carry oxygen, which is important for all of the body's functions. When there are fewer red blood cells, the body does not get all the oxygen it needs.

Kidneys remove toxins from the blood; they also measure the amount of oxygen in the blood. If there is not enough oxygen, the kidneys will produce a hormone called erythropoietin. Erythropoietin is released into the bloodstream and travels to the bone marrow where red blood cells are made. Erythropoietin signals the bone marrow to make more oxygen-carrying red blood cells.

As the kidneys fail, they stop cleansing toxins from your blood. They also make less erythropoietin than they should. Therefore, the bone marrow does not receive a strong-enough signal to make the oxygen-carrying red blood cells. Fewer red blood cells are produced so the muscles, brain, and other parts of the body do not get the oxygen they need to function properly.

Most patients treated with PROCRIT no longer need blood transfusions. However, certain medical conditions, or unexpected blood loss, may result in the need for a transfusion.

What do I need to know if I am giving myself PROCRIT injections?

When you receive your PROCRIT from the dialysis center, doctor's office or home dialysis supplier, always check to see that:

1. The name PROCRIT appears on the carton and vial label.
2. You will be able to use PROCRIT before the expiration date stamped on the package.

The PROCRIT solution in the vial should always be clear and colorless. Do not use PROCRIT if the contents of the vial appear discolored or cloudy, or if the vial appears to contain lumps, flakes, or particles. In addition, if the vial has been shaken vigorously, the solution may appear to be frothy and should not be used. Therefore, care should be taken not to shake the PROCRIT vial vigorously before use. Unless you have been prescribed Multidose PROCRIT (1 mL or 2 mL vials with a big "M" on the label, each containing a total of 20,000 Units of PROCRIT), vials of PROCRIT are for single use. Any unused portion of a vial should not be used. However, Multidose PROCRIT may be stored in the refrigerator between doses for up to 21 days, and can be used for multiple doses. Follow your dialysis center's instructions on what to do with the used vials.

How should I store PROCRIT?

PROCRIT should be stored in the refrigerator, but not in the freezing compartment. Do not let the vial freeze and do not leave it in direct sunlight. Do not use a vial of PROCRIT that has been frozen or after the expiration date that is stamped on the label. If you have any questions about the safety of a vial of PROCRIT that has been subjected to temperature extremes, be sure to check with your dialysis unit staff.

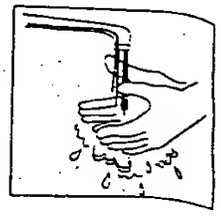
Always use the correct syringe.

Your doctor has instructed you on how to give yourself the correct dosage of PROCRIT. This dosage will usually be measured in Units per milliliter or cc's. It is important to use a syringe that is marked in tenths of milliliters (for example, 0.2 mL or cc). Failure to use the proper syringe can lead to a mistake in dosage, and you may receive too much or too little PROCRIT. Too little PROCRIT may not be effective in increasing your hematocrit, and too much PROCRIT may lead to a hematocrit that is too high. Only use disposable syringes and needles as they do not require sterilization; they should be used once and disposed of as instructed by your doctor.

IMPORTANT: TO HELP AVOID CONTAMINATION AND POSSIBLE INFECTION, FOLLOW THESE INSTRUCTIONS EXACTLY.

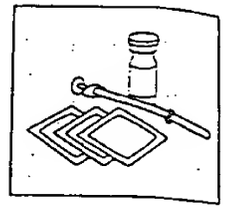
PREPARING THE DOSE

1. Wash your hands thoroughly with soap and water before preparing the medication.

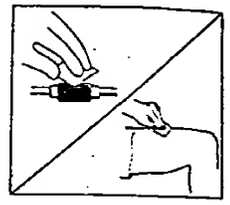


2. Check the date on the PROCRIT vial to be sure that the drug has not expired.

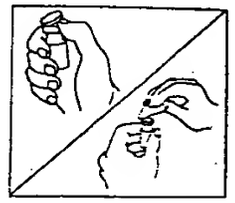
3. Remove the vial of PROCRIT from the refrigerator and allow it to reach room temperature. Each PROCRIT vial is designed to be used only once; do not reenter the vial. It is not necessary to shake PROCRIT. Prolonged vigorous shaking may damage the product. Assemble the other supplies you will need for your injection.



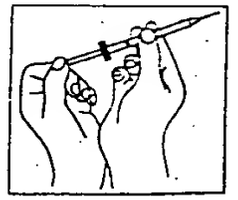
4. Hemodialysis patients should wipe off the venous port of the hemodialysis tubing with an antiseptic swab. Peritoneal dialysis patients should cleanse the skin with an antiseptic swab where the injection is to be made.



5. Flip off the red protective cap but do not remove the gray rubber stopper. Wipe the top of the gray rubber stopper with an antiseptic swab.

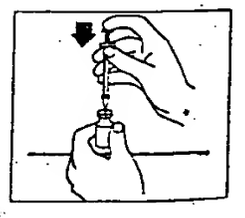


6. Using a syringe and needle designed for subcutaneous injection, draw air into the syringe by pulling back on the plunger. The amount of air should be equal to your PROCRIT dose.

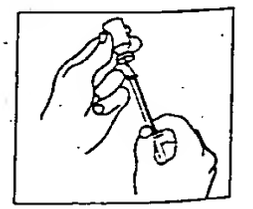


7. Carefully remove the needle cover. Put the needle through the gray rubber stopper of the PROCRIT vial.

8. Push the plunger in to discharge air into the vial. The air injected into the vial will allow PROCRIT to be easily withdrawn into the syringe.



9. Turn the vial and syringe upside down in one hand. Be sure the tip of the needle is in the PROCRIT solution. Your other hand will be free to move the plunger. Draw back on the plunger slowly to draw the correct dose of PROCRIT into the syringe.



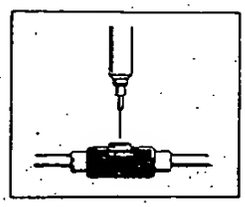
10. Check for air bubbles. The air is harmless, but too large an air bubble will reduce the PROCRIT dose. To remove air bubbles, gently tap the syringe to move the air bubbles to the top of the syringe, then use the plunger to push the solution and the air back into the vial. Then remeasure your correct dose of PROCRIT.

11. Double check your dose. Remove the needle from the vial. Do not lay the syringe down or allow the needle to touch anything.

INJECTING THE DOSE

Patients on home hemodialysis using the intravenous injection route:

. Insert the needle of the syringe into the previously cleansed venous port and inject the PROCRIT.

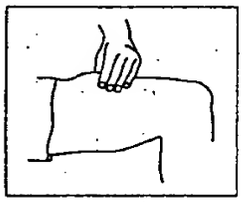


. Remove the syringe and dispose of the whole unit. Use the disposable syringe only once. Dispose of syringes and needles as directed by your doctor, by following these simple steps:

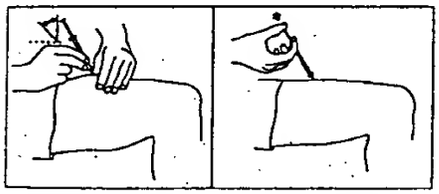
- Place all used needles and syringes in a hard plastic container with a screw-on-cap, or a metal container with a plastic lid, such as a coffee can properly labeled as to content. If a metal container is used, cut a small hole in the plastic lid and tape the lid to the metal container. If a hard-plastic container is used, always screw the cap on tightly after each use. When the container is full, tape around the cap or lid, and dispose of according to your doctor's instructions.
- Do not use glass or clear plastic containers, or any container that will be recycled or returned to a store.
- Always store the container out of the reach of children.
- Please check with your doctor, nurse, or pharmacist for other suggestions. There may be special state and local laws that they will discuss with you.

atients on home peritoneal dialysis or home hemodialysis sing the subcutaneous route:

With one hand, stabilize he previously cleansed kin by spreading it or by inching up a large area ith your free hand.



. Hold the syringe with he other hand, as you ould a pencil. Double eck that the correct ount of PROCRIT is in e syringe. Insert the edle straight into the skin 0 degree angle). Pull the plunger back slightly. If blood mes into the syringe, do not inject PROCRIT, as the edle has entered a blood vessel; withdraw the syringe and ject at a different site. Inject the PROCRIT by pushing the unger all the way down.



Hold an antiseptic swab near the needle and pull the edle straight out of the skin. Press the antiseptic swab er the injection site for several seconds.

Use the disposable syringe only once. Dispose of sy- ges and needles as directed by your doctor, by following se simple steps:

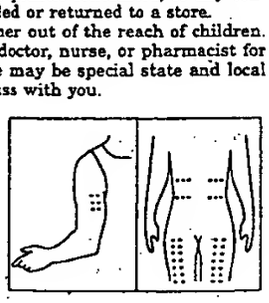
Place all used needles and syringes in a hard plastic container with a screw-on-cap, or a metal container with a plastic lid, such as a coffee can properly labeled as to content. If a metal container is used, cut a small hole in the plastic lid and tape the lid to the metal container. If a hard-plastic container is used, always screw the cap on tightly after each use. When the container is full, tape around the cap or lid, and dispose of according to your doctor's instructions.

Do not use glass or clear plastic containers, or any container that will be recycled or returned to a store.

Always store the container out of the reach of children.

Please check with your doctor, nurse, or pharmacist for other suggestions. There may be special state and local laws that they will discuss with you.

Always change the site each injection is directed. Occasionally a problem may develop at the injection site. If you notice a lump, swelling, or bruising that doesn't go away, contact your doctor. You may wish to record the site just used so that you can keep track.



USAGE IN PREGNANCY

If you are pregnant or nursing a baby, consult your doctor before using PROCRIT.

IMPORTANT NOTES

Once you are a home dialysis patient and your doctor allows you to self-administer PROCRIT, please note the following:

Always follow the instructions of your doctor concerning the dosage and administration of PROCRIT. Do not change the dose or instructions for administration of PROCRIT without consulting your doctor.

Your doctor will tell you what to do if you miss a dose of PROCRIT. Always keep a spare syringe and needle on hand.

3. Always consult your doctor if you notice anything unusual about your condition or your use of PROCRIT.

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Amgen Inc.
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Shown in Product Identification Guide, page 328

SPORANOX®

[*spōr-a'nŏx*]
(itraconazole) INJECTION

WARNING: Coadministration of terfenadine, astemizole, and cisapride with SPORANOX® (itraconazole) Capsules, Oral Solution or Injection is contraindicated. SPORANOX® is a potent inhibitor of the cytochrome P450 3A4 enzyme system and may raise plasma concentrations of drugs metabolized by this pathway. Serious cardiovascular events, including death, ventricular tachycardia, and torsades de pointes have occurred in patients taking itraconazole concomitantly with terfenadine or cisapride, which are metabolized by the cytochrome P450 3A4 system. See CONTRAINDICATIONS, WARNINGS, and PRECAUTIONS: Drug Interactions for more information.

DESCRIPTION

For Intravenous Infusion (NOT FOR IV BOLUS INJECTION)

SPORANOX® is the brand name for itraconazole, a synthetic triazole antifungal agent. Itraconazole is a 1:1:1:1 racemic mixture of four diastereomers (two enantiomeric pairs), each possessing three chiral centers. It may be represented by the following structural formula and nomenclature:

(±)-1-[(R*)-sec-butyl]-4-[p-[4-[p[[(2R*,4S*)-2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl-]methoxy]phenyl]-1-piperazinyl]phenyl]-Δ^2-1,2,4-triazolin-5-one mixture with (±)-1-[(R*)-sec-butyl]-4-[p-[4-[p[[2S*,4R*)-2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl]-
phenyl]-Δ^2-1,2,4-triazolin-5-one

or

(±)-1-[(RS*)-sec-butyl]-4-[p-[4-[p[[(2R*,4S)-2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl-]methoxy]phenyl]-1-piperazinyl]phenyl]-Δ^2-1,2,4-triazolin-5-one

Itraconazole has a molecular formula of $C_{35}H_{38}Cl_2N_8O_4$ and a molecular weight of 705.64. It is a white to slightly yellowish powder. It is insoluble in water, very slightly soluble in alcohols, and freely soluble in dichloromethane. It has a pKa of 3.70 (based on extrapolation of values obtained from methanolic solutions) and a log (n-octanol/water) partition coefficient of 5.66 at pH 8.1.

SPORANOX® (itraconazole) Injection is a sterile pyrogen-free clear, colorless to slightly yellow solution for intravenous infusion. Each mL contains 10 mg of itraconazole, solubilized by hydroxypropyl-β-cyclodextrin (400 mg) as a molecular inclusion complex, with 3.8 µL hydrochloric acid, 25 µL propylene glycol, and sodium hydroxide for pH adjustment to 4.5, in water for injection. SPORANOX® Injection is packaged in 25 mL colorless glass ampules, containing 250 mg of itraconazole, contents of which are diluted in 50 mL 0.9% Sodium Chloride Injection, USP (Normal Saline) prior to infusion. When properly administered, contents of one ampule will supply 200 mg of itraconazole.

CLINICAL PHARMACOLOGY

Pharmacokinetics and Metabolism: NOTE: The plasma concentrations reported below were measured by high performance liquid chromatography (HPLC) specific for itraconazole. When itraconazole in plasma is measured by a bioassay, values reported may be higher than those obtained by HPLC due to the presence of the bioactive metabolite, hydroxyitraconazole. (See MICROBIOLOGY.)

The pharmacokinetics of SPORANOX® (itraconazole) Injection (200 mg b.i.d. for two days, then 200 mg q.d. for five days) followed by oral dosing of SPORANOX® Capsules were studied in patients with advanced HIV infection. Steady-state plasma concentrations were reached after the fourth dose for itraconazole and by the seventh dose for hydroxyitraconazole. Steady-state plasma concentrations were maintained by administration of SPORANOX® Capsules, 200 mg b.i.d. Pharmacokinetic parameters for itraconazole and hydroxyitraconazole are presented in the table below: [See table below]

The estimated mean ±SD half-life at steady state of itraconazole after intravenous infusion was 35.4 ± 29.4 hours. In previous studies, the mean elimination half-life for itraconazole at steady state after daily oral administration of 100 to 400 mg was 30–40 hours. Approximately 93–101% of hydroxypropyl-β-cyclodextrin was excreted unchanged in the urine within 12 hours after dosing.

The plasma protein binding of itraconazole is 99.6% and that of hydroxyitraconazole is 99.5%. Following intravenous administration, the volume of distribution of itraconazole averaged 796 ± 185 L.

Itraconazole is extensively metabolized resulting in the formation of several metabolites including hydroxyitraconazole, the major metabolite. Results of a pharmacokinetics study suggest that itraconazole may undergo saturable metabolism with multiple dosing. Fecal excretion of the parent drug varies between 3–18% of the dose. Renal excretion of the parent drug is less than 0.03% of the dose. About 40% of the dose is excreted as inactive metabolites in the urine. No single excreted metabolite represents more than 5% of a dose. Itraconazole total plasma clearance averaged 381 ± 95 mL/min following intravenous administration. Approximately 80–90% of hydroxypropyl-β-cyclodextrin is eliminated through the kidneys.

Special populations:

Renal Insufficiency: Plasma concentrations of itraconazole in patients with mild to moderate renal insufficiency were comparable to those obtained in healthy subjects. The majority of the 8-gram dose of hydroxypropyl-β-cyclodextrin was eliminated in the urine during the 120-hour collection period in normal subjects and in patients with mild to severe renal insufficiency. Following a single intravenous dose of 200 mg to subjects with severe renal impairment (creatinine clearance ≤ 19 mL/minute), clearance of hydroxypropyl-β-cyclodextrin was reduced six-fold compared with subjects with normal renal function. SPORANOX® Injection should not be used in patients with creatinine clearance < 30 mL/min.

Hepatic Insufficiency: The effect of hepatic impairment on plasma concentrations of itraconazole is unknown. It is recommended that patients with hepatic impairment be carefully monitored when taking itraconazole.

MICROBIOLOGY

Mechanism of Action: *In vitro* studies have demonstrated that itraconazole inhibits the cytochrome P-450-dependent synthesis of ergosterol, which is a vital component of fungal cell membranes.

Activity in vitro and in vivo: Itraconazole exhibits *in vitro* activity against *Blastomyces dermatitidis, Histoplasma capsulatum, Histoplasma duboisii, Aspergillus flavus, Aspergillus fumigatus, Candida albicans* and *Cryptococcus neoformans.* Itraconazole also exhibits varying *in vitro* activity against *Sporothrix schenckii,* Trichophyton spp., *Candida krusei* and other Candida spp. The bioactive metabolite, hydroxyitraconazole, has not been evaluated against *Histoplasma capsulatum* and *Blastomyces dermatitidis.* Correlation between *in vitro* minimum inhibitory concentration (MIC) results and clinical outcome has yet to be established for azole antifungal agents.

Itraconazole administered orally was active in a variety of animal models of fungal infection using standard laboratory strains of fungi. Fungistatic activity has been demonstrated against disseminated fungal infections caused by *Blastomyces dermatitidis, Histoplasma duboisii, Aspergillus fumigatus, Coccidioides immitis, Cryptococcus neoformans, Paracoccidioides brasiliensis, Sporothrix schenckii, Trichophyton rubrum* and *Trichophyton mentagrophytes.*

Itraconazole administered at 2.5 mg/kg and 5.0 mg/kg via the oral and parenteral routes increased survival rates and sterilized organ systems in normal and immunosuppressed guinea pigs with disseminated *Aspergillus fumigatus* infections. Oral itraconazole administered daily at 40 mg/kg and 80 mg/kg increased survival rates in normal rabbits with disseminated disease and immunosuppressed rats with pulmonary *Aspergillus fumigatus* infection, respectively. Itraconazole has demonstrated antifungal activity in a variety of animal models infected with *Candida albicans* and other Candida species.

Resistance: Isolates from several fungal species with decreased susceptibility to itraconazole have been isolated *in vitro* and from patients receiving prolonged therapy.

Several *in vitro* studies have reported that some fungal clinical isolates, including Candida species, with reduced susceptibility to one azole antifungal agent may also be less susceptible to other azole derivatives. The finding of cross-resistance is dependent upon a number of factors, including the species evaluated, its clinical history, the particular

Continued on next page

Parameter	Injection Day 7 n = 29		Capsules, 200 mg b.i.d. Day 36 n = 12	
	itraconazole	hydroxyitraconazole	itraconazole	hydroxyitraconazole
C_{max} (ng/mL)	2856 ± 866*	1906 ± 612	2010 ± 1420	2514 ± 1703
t_{max} (hr)	1.08 ± 0.14	8.53 ± 6.36	3.92 ± 1.83	5.92 ± 6.14
AUC_{0-12h} (ng·h/mL)	—	—	18768 ± 12933	28516 ± 19149
AUC_{0-24h} (ng·h/mL)	30605 ± 8961	42445 ± 13282	—	—

*mean ± standard deviation

Attachments JJF 18

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REGULAR PAPER

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Effect of transient focal ischemia on blood-brain barrier permeability in the rat: correlation to cell injury

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Abstract Prolonged ischemia is known to damage the blood-brain barrier, causing an increase in vascular permeability to proteins. We studied the time course of extravasation of endogenous albumin in rats after 1 and 2 h of middle cerebral artery (MCA) occlusion followed by 6, 12, and 24 h of recirculation. In a separate group of rats that had undergone 1 h of MCA occlusion and 6 h of recirculation, influx of [^{14}C]aminoisobutyric acid (AIB) from blood to brain was also measured. After 1 h of occlusion followed by 6 h of recirculation, neuronal damage was evident in caudoputamen, but there were no signs of blood-brain barrier leakage to either AIB or albumin. At 12 h, the caudoputamen contained extravasated albumin, and at 24 h extravasation was extended to the somatosensory cortex. Animals subjected to 2 h of MCA occlusion showed albumin extravasation in caudoputamen already at 6 h of recirculation, and at 12 and 24 h albumin was abundant in the major part of the right hemisphere. This study suggests that damage to neurons precedes leakage of the blood-brain barrier. Even a relatively short period of ischemia such as 1 h will result in markedly increased vascular permeability. However, a longer transient ischemic insult disrupts the blood-brain barrier earlier than a shorter one.

Key words Brain · Focal ischemia · Reperfusion · Albumin extravasation · Blood-brain barrier

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Introduction

Several groups have investigated the integrity of the blood-brain barrier during focal ischemia [4, 7, 14, 20, 23, 26]. In cases of stroke early edema is of the cytotoxic type, but permanent focal ischemia of longer than 6 h duration will also damage the blood-brain barrier [16, 26] and give rise to an increase in vascular permeability to large molecules, such as proteins. When the ischemia is transient, vascular dysfunction appears earlier, and has been shown to be more severe [7, 20, 26]. An open question is whether endothelial cell damage, and thereby blood-brain barrier dysfunction, precedes neuronal injury after transient ischemia, or if primary ichemic cell damage is followed by loss of vascular integrity.

In this study, we examined the integrity of the blood-brain barrier after transient focal ischemia of short duration (1–2 h), and explored whether there was a correlation between the development of tissue damage and blood-brain barrier dysfunction. For this, we studied the time course for extravasation of endogenous albumin after 1 and 2 h of middle cerebral artery (MCA) occlusion followed by 6, 12, and 24 h of recirculation, and evaluated morphological tissue damage in the ischemic focus (lateral caudoputamen and somatosensory cortex), and the penumbral area (motor cortex).

Materials and methods

Animals and operative techniques

Fasted male Wistar rats (Møllegaard's Breeding Center, Copenhagen, Denmark) were used for this study. The NIH principles of laboratory care were followed, and the experiments were approved by the Ethical Committee for Laboratory Animal Experiments at the University of Lund. Focal ischemia was induced by occlusion of the right middle cerebral artery with an intraluminal filament [15, 17–19] under halothane anesthesia. The animals were allowed to recover from anesthesia, and adequate occlusion of the MCA was confirmed by the occurrence of a neurological deficit, with the animals circling to the left [2]. After 1 or 2 h blood flow was restored to the ischemic area by withdrawal of the occluding fila-

ment under renewed anesthesia. The animals were thereafter allowed recirculation periods of 6, 12, or 24 h.

Immunohistochemistry

At the end of the recirculation period (6, 12, and 24 h, $n = 8$ in each group), the animals were again anesthetized, and the brains were perfusion-fixed with formaldehyde, sectioned coronally in 2.8-mm-thick blocks, dehydrated, and embedded in paraffin. Coronal brain sections of 5-µm thickness taken at the level of bregma, the region which usually shows the largest infarct area in this model, were used for immunohistochemical visualization of extravasated serum albumin using the avidin-biotin-peroxidase complex (ABC) method [13]. In brief, the sections were placed on chrome-gelatine-covered glass slides, deparaffinized, and rehydrated. Thereafter, the sections were rinsed in TRIS-HCl buffer solution (TBS), and incubated in 0.4% pepsin in TBS for 15 min at 37°C to unmask antigen sites, followed by rinsing in TBS. To eliminate endogenous peroxidase activity, the sections were incubated for 30 min with 0.3% hydrogen peroxide in methanol. To reduce the nonspecific protein binding, the sections were incubated for 2 h at room temperature with 1% chicken egg albumin in TBS. The sections were then incubated for 48 h at 4°C with the primary antibody, rat albumin antiserum, diluted to 1:8000 in 1% chicken egg albumin, followed by incubation with the biotinylated secondary antibody and ABC (Vectastain, ABC-kit, Vector Laboratories, Burlingame, Calif.). The bound antigen-antibody-ABC complex was visualized using 3,3'-diaminobenzidine as a chromogen. The sections were counterstained with hematoxylin-eosin, and evaluated by light microscopy.

Aminoisobutyric acid penetration

Four animals subjected to 1 h MCA occlusion followed by 6-h recirculation were re-anesthetized, and the integrity of the blood-brain barrier to aminoisobutyric acid (AIB) was tested as follows. A bolus of 15 µCi [^{14}C]AIB diluted in 1.0 ml saline was injected i.v., and was allowed to circulate for 20 min. Arterial plasma samples (20 µl) were repeatedly collected to allow integration of specific activity. At the end of the experiment, the animals were decapitated, and their brains were removed and frozen in 2-methylbutane chilled to –50°C. Tissue samples, weighing 10–15 mg, were dissected at –15°C from the ischemic focus (lateral caudoputamen) and penumbra (motor cortex) of the MCA-occluded hemisphere (Fig. 1) to allow assessment of tissue tracer activity.

The plasma-to-brain AIB transfer coefficient, K (µl · g/min) was calculated as described by Blasberg et al. [5] using the following equation:

$$K = \frac{C^*_{br}(T)}{\int_0^T C^*_{pl}(t)\,dt}$$

in which C^*_{br} is the brain tissue concentration of radioactivity after 20 min, and C^*_{pl} is the plasma activity. The cerebral blood volume was set to 0.01 ml/g tissue, according to Ohta et al. [21], to allow correction for AIB remaining in the vasculature.

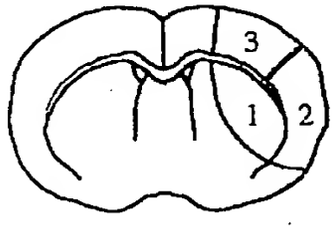

Fig. 1 Schematic drawing of the section used for histological evaluation of albumin extravasation. *1* Lateral caudoputamen (ischemic focus), *2* cortical focus, *3* cortical penumbra. Tissue samples for measurement of aminoisobutyric acid influx were taken from areas *1* and *3*

Results

Physiological parameters

Blood pressure, body temperature, blood gases and pH were measured just before and 10 min after the MCA occlusion, and were found to be within the normal ranges in all animals.

Albumin leakage

Three patterns of albumin incorporation into tissue were seen, all of which were usually present in the same animal: (1) vague neuropil staining, evenly distributed in the damaged area; (2) normally shaped neurons taking up albumin in the cytoplasm; and (3) shrunken necrotic neurons containing albumin in the whole cell.

Albumin leakage was mainly confined to the ischemic area, with the earliest changes seen in the ischemic focus, while prolongation of recirculation caused the changes to spread into penumbral tissues. After 24 h of recirculation the area of albumin extravasation and the area containing necrotic neurons/infarction were closely overlapping. In the 1-h MCA occlusion group, neuronal damage preceded albumin leakage in the ischemic focus.

Caudoputamen, 1-h MCA occlusion

No sign of blood-brain barrier leakage to albumin was found after 6 h of recirculation, but neuronal damage was evident to a mild to moderate extent (Fig. 2A). After 12 h of recirculation, the caudoputamen contained albumin, mainly distributed around vessels, and taken up by dying neurons (Fig. 2B). After 24 h of recirculation all neurons, including the still normal-shaped, large caudoputaminal neurons, contained albumin (Fig. 2C).

Cortex, 1-h MCA occlusion

After 6 h of recovery the neocortex had a completely normal appearence; after 12 h two animals were still unaf-

Fig. 2A–D Photomicrographs from caudoputamen, stained for visualization of endogenous albumin in tissue. A After 1-h MCA occlusion plus 6 h of recirculation, many shrunken, damaged neurons (some marked by *arrowheads*), but no albumin staining, are seen. B After 1-h MCA occlusion plus 12 h of recirculation, albumin is visible as brown staining in the neuropil around the vessel (*V*), and has been taken up by damaged neurons (some marked with *arrows*). C After 1-h MCA occlusion plus 24 h of recirculation, all neurons in the caudoputaminal focus have incorporated albumin and show still normal-shaped large neurons, known to be less sensitive to ischemia (*arrow*). D After 2-h MCA occlusion plus 6 h of recirculation, i.e., after a longer insult, albumin uptake is already seen in neurons showing ischemic cell changes (some marked with *arrows*). A–D Hematoxylin-eosin plus the avidin-biotin-peroxidase (ABC) method for albumin staining. *Bar* = 50 µm

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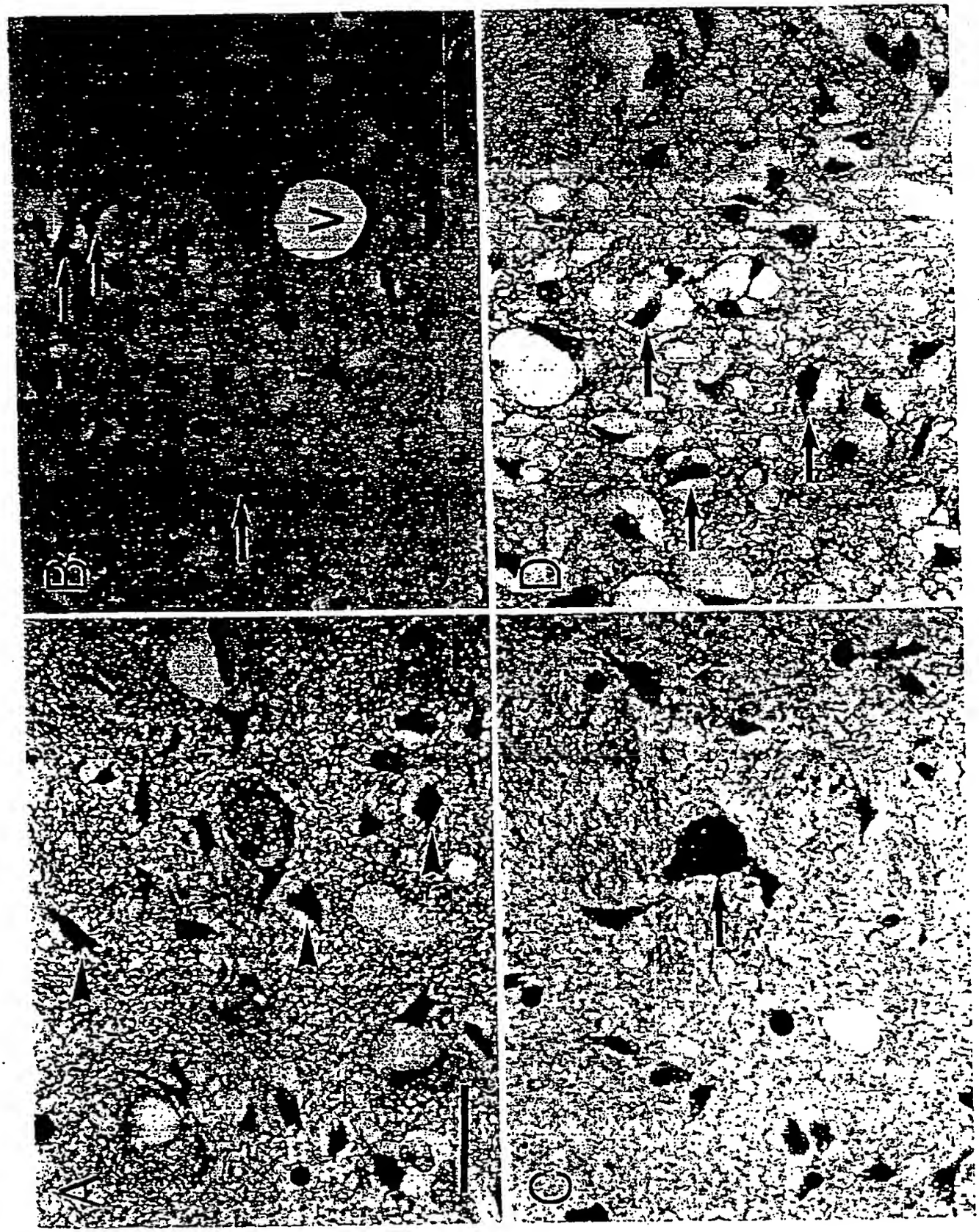

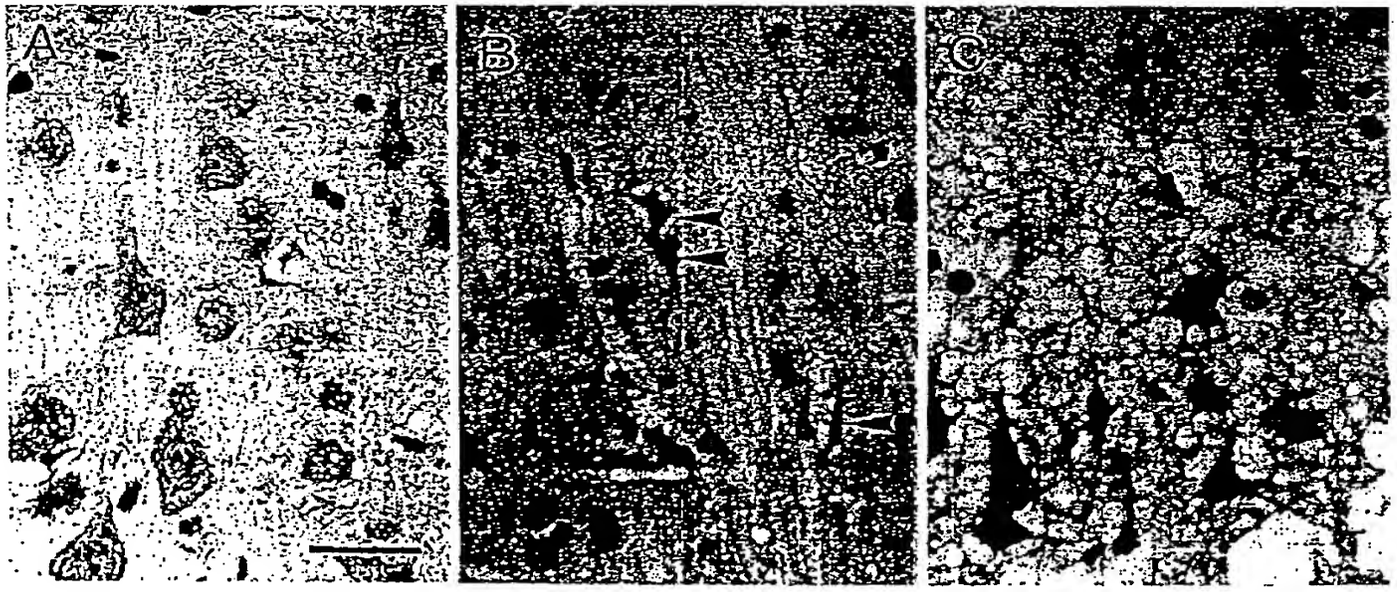

Fig. 3A–C Photomicrographs of the cortical penumbra at different times of recirculation after 2 h of MCA occlusion. A No injured neurons and no extravasation of albumin is seen at 6 h of recirculation. B At 12 h of recirculation the neuropil show a diffuse staining for albumin, and some neurons exhibit ischemic cell changes without albumin incorporation (*arrowheads*). C After 24 h of recirculation all tissue components in the severely damaged tissue contain albumin. A–C Hematoxylin-eosin plus the ABC method for albumin staining. *Bar* = 50 μm

fected and the remaining six showed pale albumin staining and necrotic neurons in the somatosensory cortex (cortical focus). The pattern after 24 h of recirculation was similar to that at 12 h, with two animals without detectable damage or albumin extravasation, and the remaining exhibiting diffuse albumin staining and damaged neurons often containing albumin.

Caudoputamen, 2-h MCA occlusion

Albumin extravasation was seen already at the earliest recirculation time studied, 6 h. Neuronal damage in the lateral caudoputamen was dense, and the shrunken neurons contained albumin (Fig. 2D). After 12 and 24 h, serum albumin was abundant in both neuronal and glial compartments in this structure (not shown).

Cortex, 2-h MCA occlusion

The somatosensory cortex, directly overlying the caudoputaminal ischemic focus, constitutes the cortical focus which during ischemia suffers a blood flow reduction similar to that of caudoputamen. Changes in the somatosensory cortex followed a pattern similar to that seen in the caudoputamen after 2-h ischemia, with early albumin extravasation and the presence of necrotic neurons.

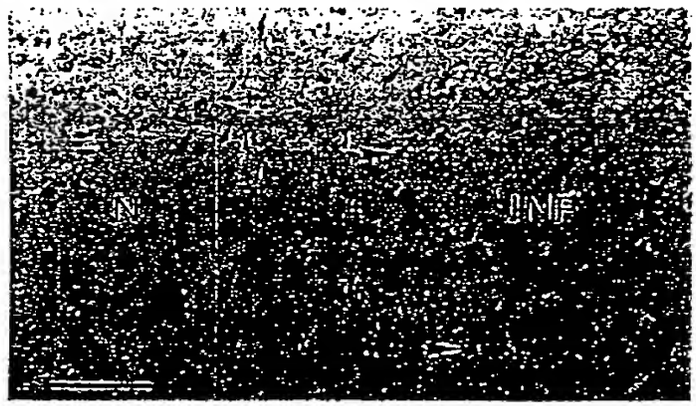

Fig. 4 Cortex at 24 h of recirculation following 2 h of MCA occlusion: the border between normal tissue (*N*) and infarct (*INF*) is sharply demarcated both in terms of tissue damage and of albumin content. Note, however, that several damaged neurons in the borderline do not contain albumin (*arrowheads*). Hematoxylin-eosin plus the ABC method for albumin staining. *Bar* = 100 μm

In the cortical area with less dense ischemia during the MCA occlusion (the penumbral zone, see Fig. 1), no injured neurons and no extravasation of albumin were seen at 6 h after 2-h transient focal ischemia (Fig. 3A). After 12 h of recirculation some neurons showed ischemic cell changes, mainly without incorporation of albumin, and the neuropil had started to show a diffuse staining for albumin. After 24 h of recirculation the whole tissue was severely damaged and all tissue components contained albumin with the exception of a few glial cells (Fig. 3C). At this time, the border of the infarct was very demarcated both in terms of tissue damage and of albumin content (Fig. 4).

[^{14}C]Aminoisobutyric acid transfer

The transfer coefficient (K) for AIB from blood to brain after 6 h of recirculation following 1 h of MCA occlusion was 0.82 ± 0.54 and 1.23 ± 0.86 µl · g/min in the ischemic focus and penumbra, respectively. These values were not statistically different from those measured in normal control animals in corresponding areas (0.92 ± 0.14 and 1.07 ± 0.34 µl · g/min).

Discussion

In this study, despite the manifest neuronal damage, the blood-brain barrier was still intact to serum albumin, as well as to smaller molecules such as AIB, at 6 h after a transient focal ischemia of 1-h duration. With longer recirculation times (12 h) leakage to albumin became evident in the brain areas showing tissue damage.

When the duration of ischemia was prolonged to 2 h, albumin extravasation was seen already after 6 h of recirculation in tissues subjected to dense ischemia, i.e., the caudoputamen and somatosensory cortex, and exhibiting pronounced neuronal necrosis, while in the cortical penumbral area albumin was not extravasated until neuronal damage started to occur.

These findings lead us to conclude that it is unlikely that a dysfunction of the blood-brain barrier, to an extent which allows large molecules to penetrate, is involved in the development of neuronal necrosis.

Several studies have shown that the blood-brain barrier is intact to proteins, and also to smaller molecules such as AIB, for 4–6 h after permanent focal ischemia [3, 4, 16, 22–24]. Ischemic edema is formed before this time, however, due to an increased influx of sodium and chloride into the tissue. When reperfusion is instituted after 2–3 h of focal ischemia, vascular dysfunction is aggravated, and vasogenic edema ensues, adding to an already increased water content of the tissue [7, 16, 20, 26].

Our results demonstrate that the blood-brain barrier permeability to albumin increases at a time when neuronal damage is already manifest. A morphological study by Garcia et al. [11] supports this finding, in showing that neuronal necrosis becomes prominent 6 h after permanent MCA occlusion in rats, i.e., at a time point at which the blood-brain barrier is intact [4, 26]. Furthermore, Tamura et al. [25] studying focal ischemia in cats, demonstrated that only animals with severe tissue damage showed blood-brain barrier disruption, while subjects with mild or no damage had an intact barrier to Evans blue. However, the extent of damage in that study was very severe, including hemorrhagic infarcts in three out of four cats with Evans blue leakage, rendering the information less conclusive. Thus, to our knowledge, the present study is the first to demonstrate that neuronal damage precedes, and even may be a prerequisite for a blood-brain barrier opening following transient focal ischemia.

The mechanisms behind the breakdown of the barrier following ischemia remains unclear, but an improvement of barrier stability has been shown in situations which are associated with nerve cell protection, such as treatment with a nitric oxide synthase inhibitor [20], as well as with hypothermia [14], while aggravation of blood-brain barrier dysfunction has been seen as an effect of platelet-activating factor [6] and arachidonic acid metabolites [1, 7]. Taken together, these studies suggest that the cascade of events following massive glutamate release during ischemia is ultimately involved in creating endothelial cell damage. The cellular origin of deletarious molecules formed by such mechanisms, and exerting their action on the vasculature, is not known. They may be formed in neural tissue or in vascular cells. In the latter case, aggregation of platelets, and expression of adhesion molecules causing leukocyte sticking to the vessel wall ([9], for reviews see [8, 10, 12]), may cause microcirculatory disturbances and further increase endothelial injury. However, more research is needed to determine whether the endothelial damage originates from injury to neural tissue or to the vasculature.

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Beneficial Effects of Lysine Acetylsalicylate, a Soluble Salt of Aspirin, on Motor Performance in a Transgenic Model of Amyotrophic Lateral Sclerosis

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We have studied the effect of lysine acetylsalicylate (LAS; Aspegic), a soluble salt of aspirin, on motor deficits in transgenic mice expressing a human superoxide dismutase SOD1 mutation (Gly-93 → Ala), an animal model of familial amyotrophic lateral sclerosis (FALS). In nontreated FALS mice, motor impairments appear at 12–14 weeks of age, whereas paralysis is not observed before 20 weeks of age. Life expectancy is 140–170 days. Early treatment with LAS from 5 weeks of age delayed the appearance of motor deficits in FALS mice as measured by extension reflex, loaded grid, and rotarod tests. This beneficial effect of treatment was maintained up to 18 weeks of age, until just before onset of end-stage disease. When treatment was started at 13 weeks, no significant beneficial effect was observed. These results demonstrate that chronic LAS treatment is able to delay the appearance of reflex, coordination, and muscle strength deficits in this animal model of ALS if the treatment is started early enough. However, neither the onset of paralysis nor end-stage disease were improved by the LAS treatment. In the absence of an effect on survival, the functional improvement demonstrated here is probably the maximum that this demanding model could allow. Although other properties of LAS may have contributed to its beneficial effect, we suggest that the antioxidant properties of aspirin are responsible for the positive effects in this model and support the use of antioxidants as effective therapy for ALS. © 1999 Academic Press

Key Words: transgenic mice; neuroprotection; antioxidant; aspirin; behavior.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder which is characterized by the loss of motor neurons in the cerebral cortex, brain stem, and spinal cord (for review see (9)). The affected neurons demonstrate evidence of cytoskeletal pathology in the form of accumulations of neurofilaments (26). A number of studies suggest that this disease arises from oxidative and excitotoxic injury to critical subcellular targets in motor neurons (6, 8). For example, an 85% increase in protein carbonyl groups, a marker for oxidative damage, has been reported in brain tissue from cases of sporadic ALS (8). In addition, a series of mutations in the CuZn-superoxide dismutase-1 gene (SOD1), located on chromosome 21, have been identified in 15 to 20% of familial ALS (FALS) cases (17, 37), and Wiedau-Pazos *et al.* (42) and Yim *et al.* (44) have demonstrated that oxidative reactions catalyzed by such mutant SOD1 enzymes can initiate the neuropathological changes in FALS.

To test the hypothesis that FALS results from a direct action of the mutant enzyme, transgenic mice that express mutant forms of human SOD1 have been generated (25, 36, 43). These studies demonstrate that mice with a high number of gene copies become paralyzed as a result of motor neuron loss from the spinal cord and die at 5 to 6 months of age. Electromyographic studies have demonstrated that the function of motor neurons in these mice becomes impaired from 7 weeks of age (28), and the onset of motor neuron death is observed from 13 weeks (13). Several studies suggest that neuronal degeneration in these transgenic mice is mediated by free radical production. An increase of lipid peroxidation has been demonstrated in the spinal cord of these transgenic mice (1, 35), and chronic treatment with the antioxidant vitamin E can delay the onset of symptomatology (24).

There are several compelling reasons for testing salicylate and its derivatives in neurodegenerative disorders. First, it has high potency to scavenge hydroxyl radicals as demonstrated by its use in assay systems (14). Such an activity has been suggested *in vivo* in a recent study by Aubin *et al.* (2), who demonstrated the ability of salicylate to block the effects of

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MPTP in mice. Second, salicylate was recently reported to protect against glutamate neurotoxicity via blockade of NF-κB activation (22). Finally, salicylate is readily available and has good brain penetration. Both free radicals and glutamate toxicity have been suggested to play a role in motor neurodegeneration (5, 18, 19). We have therefore studied the effects of a soluble salicylate derivative, lysine acetyl salicylate (LAS, Aspegic), on motor performance and survival of FALS mice. Two treatment regimes were used starting at 5 weeks of age (i.e., before the onset of motor neuron dysfunction) and at 13 weeks of age (i.e., the onset of motor neuron death).

MATERIALS AND METHODS

Transgenic Mice

Transgenic male mice expressing mutant human CuZn-SOD-1 with a $gly^{93} \rightarrow ala$ substitution (designated B6SJL-TgN(SOD1-G93A)1Gur) were bred and maintained by Transgenic Alliance (Saint-Germain-sur l'Arbresle, France). The transgenic mouse line was maintained as hemizygote by mating transgenic males with B6 females. Transgenic progeny were identified by polymerase chain reaction (PCR) amplification of tail DNA (25). Nontransgenic wild-type littermates (wt) were used as controls of FALS transgenic mice. A total of 153 FALS and wt mice were included in two differents experiments (see Table 1). Ninety-seven mice were used for the first experiment in which the effects of LAS on motor deficits and survival were studied; the FALS animals were subdivided in FALSO (no treatment), FALS5 (onset of treatment at 5 weeks of age), and FALS13 (onset of treatment at 13 weeks of age).

TABLE 1

Details of the Experimental Protocols and Numbers of Animals Used in Experiments 1 and 2

Group	Strain	Treatment	Age at start of treatment	Age at start of behavior	Duration testing of treatment	Number tested
			Experiment 1			
FALS0	FALS	Water	—	7	—	18
wt0	wt	Water	—	7	—	13
FALS5	FALS	LAS	5	7	15–19	17
wt5	wt	LAS	5	7	15–19	16
FALS13	FALS	LAS	13	7	7–11	20
wt13	wt	LAS	13	7	7–11	13
			Experiment 2			
—	FALS	water	—	—	—	14
—	wt	water	—	—	—	14
—	FALS	LAS	5	—	3	14
—	wt	LAS	5	—	3	14

Note. Values are expressed as weeks.

Fifty-six mice were used for the second experiment in which brain salicylate levels after the free consumption of LAS solution were evaluated. Mice were housed in groups of 5–7 in macrolon cages (32 × 21 × 14 cm) and kept in an isolation cabinet. They were maintained on a 12-h dark–light cycle (lights on 7:00 a.m.). Throughout the experiment food was placed on the floor of the cage so that mice could easily eat despite appearance of disease symptoms and the water bottle spout was also placed close to the floor. Body weight was measured twice a week.

Treatment Protocol (Table 1)

Experiment 1: Effects of LAS treatment on motor deficits in FALS mice. LAS was dissolved in drinking water at a concentration of 1.8 mg/ml (1.0 mg/ml of aspirin). The 97 male FALS and wt mice were subdivided into three treatment groups: early, in which treatment started at 5 weeks of age; late, in which treatment started at 13 weeks of age; and a control water-treated group. Daily fluid intake of water or LAS solution was estimated by weighing the drinking bottles twice a week, i.e., when the solution was changed (we checked the stability of the salicylate solution up to 1 week after preparation of the solution, as indicated below).

Experiment 2: Salicylate measurements (in the brain and in the consumed water). In order to estimate the stability of the solution, samples were analyzed up to 1 week after the preparation of the solution. To evaluate the passage of salicylate into the brain, 14 FALS and 14 wt mice were given access to the lysine acetylsalicylate solution for 3 weeks (see Table 1 for the treatment schedule). The day before sacrifice, the animals were deprived of drinking water. Three hours before sacrifice, the animals had access to either water or lysine acetylsalicylate. It was necessary to control the period of time preceding sacrifice during which the animals had access to drug solution because brain salicylate was no longer detectable 3 h after an i.p. administration of LAS (unpublished observation). Again, intake of water or LAS solution was estimated by weighing the drink bottles before and after the 3-h period. Finally, the brains were removed and stored at −80°C until analysis. Frozen left hemispheres were sonicated in 500 µl of 0.05 M $HClO_4$ containing 0.5 mM of EDTA (LABOSI) and 2 mM sodium metabisulfite. After centrifugation, the supernatant was diluted in 12 vol of HClO4-EDTA solution and 50 µl were injected onto the liquid chromatography column using a refrigerated (4°C) autoinjector Wisp 712 (Waters, Milford, MA). Separation was achieved at room temperature. The high pressure liquid chromatography (HPLC) system consisted of a pump, a stainless separation column (0.46 × 7 cm) packed with an Ultrasphere XL ODS C18, 3-µm particle size (Beckman, Fullertone, CA). The

mobile phase contained 0.05 M NaH_2PO_4, 2.5 mM octane sulfonic acid, 4% CH_3CN, pH 3. The flow rate was 0.9 ml/min. The detection was carried out by means of a Coulometric detector (model 5100A; ESA, Bedford, MA) equipped with dual analytical cells set at 0.25 and 0.75 V. Concentrations of each compound were calculated using a computing integrator (Maxima, Waters) with reference calibration curves obtained after injection of standards.

Behavioral Testing

The following tests were used to assess motor performance (for more details (4)). All the animals were studied weekly using the loaded grid, rotarod, and extension reflex tests. The first session began at 7 weeks of age. For the Medinaceli test, the animals were studied at 15, 18, and 20 weeks of age.

Loaded grid. The mouse was allowed to grip a small loaded grid weighing either 40, 30, 20, or 10 g and was then lifted by the tail. A maximum period of 30 s was allowed for each weight. The time during which the mouse was able to carry the grid loaded with each of the four weights was measured.

Rotarod. The period for which a mouse could remain on a rotating axle (3.6 cm diameter; speed of rotation, 16 rpm) without falling was measured. The test was stopped after an arbitrary limit of 180 s.

Extension reflex. An extension reflex of the hindlimbs is normally observed when a mouse is suspended in the air by its tail. However, in mice with motor neuron disease, a retraction of the hindlimb is more commonly seen. A score of 2 corresponded to a normal extension reflex of both hindpaws, a score of 1 to the extension reflex of only one hindpaw, and a score of 0 to the absence of any hindlimb extension.

Medinaceli test. Paralysis of hindlimbs was measured in mice using the test described by de Medinaceli *et al.* (16). The hind paws were dipped in ink and the mice were allowed to walk over a strip of paper in a runway (400 × 45 mm). The average stride length was measured for each mouse.

End-Stage Disease

The end-stage criterion corresponds to the motor score 2 defined by Kerasidis *et al.* (29) as "frequent and/or vigorous movement of hindlimbs but no weight bearing."

Statistical Analysis

The data from each experiment were analyzed by the appropriate analysis of variance model. To evaluate the consequence of the SOD mutation on behavior, only the nontreated FALS and wt mice were compared. To estimate the effects of the treatment, only the treated and nontreated FALS mice were compared. Because of the absence of an effect of the LAS treatment on wt mice behaviors, all the wt mice were pooled as the wt group and their behavioral response was shown as the optimal performance for each test. When required, significant interactions were analyzed by Newman–Keuls test. The effect of treatment on survival was evaluated by survival analysis using the LogRank test.

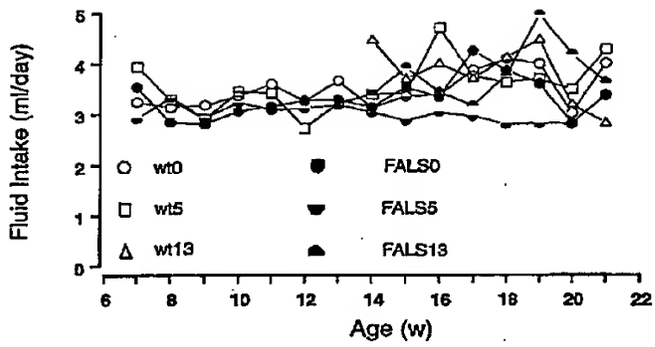

FIG. 1. Daily fluid intake for the LAS- and water-treated wt and FALS mice (experiment 1). Values are expressed as mean daily consumption per animal. See Table 1 for the details of treatment group and for the number of animals per group.

RESULTS

LAS Consumption, Treatment Tolerance, and Salicylate Brain Level Determination

Experiment 1. Daily fluid intake was stable in all groups throughout the experiment (see Fig. 1). Moreover, the presence of LAS and its derivatives did not decrease fluid intake. All the mice drunk approximately 3–4 ml of solution per day, corresponding to an aspirin dose of 3–4 mg/day (approx. 100–160 mg/kg/day). The LAS treatment did not modify body weight, again indicating the absence of toxicity of LAS in our experimental conditions (see Fig. 2).

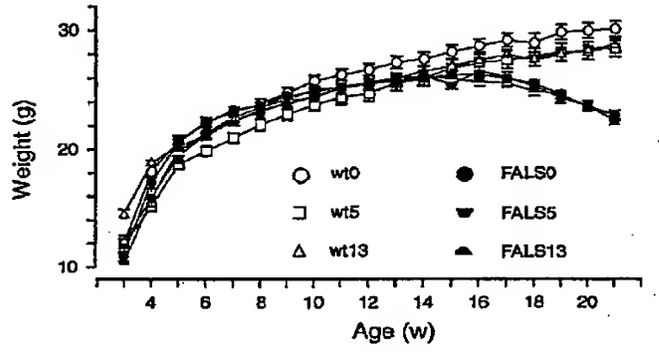

FIG. 2. Evolution of body weight in the experimental groups. Note the absence of deleterious effect of LAS on body weight. Values are expressed as mean and SEM. See Table 1 for the details of treatment group and for the number of animals per group.

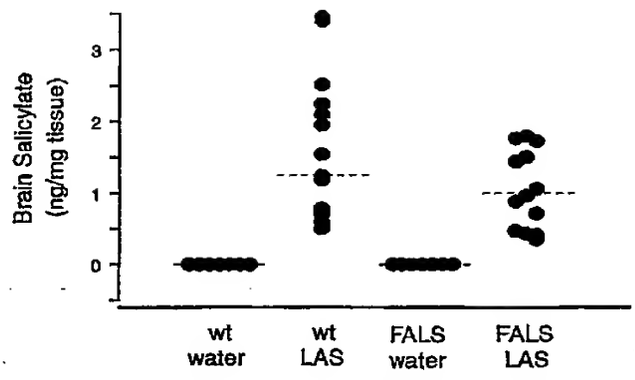

FIG. 3. Brain concentrations of salicylate in treated or nontreated mice (experiment 2). The dashed lines indicate the median value. See Table 1 for the details of treatment group and for the number of animals per group.

Experiment 2. After overnight water deprivation, the mice consumed about 2 ml of solution (2 mg of aspirin) in the 3-h period during which water or treatment was available. Salicylate was detected in the brain of LAS-treated mice at similar concentrations in wt and FALS mice. No salicylate was detected in mice drinking only water (see Fig. 3).

Effects of LAS Treatment on Motor Deficits and Survival

Muscle strength (Fig. 4). Muscular strength was estimated by the loaded grid test. As illustrated in Fig. 4, the young wt mice (7 weeks of age) had more difficulty gripping the heaviest grid than the lightest one (from 40 to 10 g, respectively). As they became older, their performance with the heaviest weights improved. In contrast, FALS mutant mice demonstrated a progressive impairment of muscle strength. There was no difference between the mutant mice and wt mice at 7 weeks of age (ANOVA one-way with group factor: $F(1,29) = 0.59$, n.s.). However, at 15 weeks of age, these two groups differed in their ability to grip the heaviest grids (ANOVA two-ways with group × weight factors interaction: $F(3,87) = 16.77$, $P < 0.001$). From 18 weeks of age, the performance of mutant mice was dramatically impaired whatever the weight of the loaded grid (ANOVA one-way with group factor: $F(1,29) = 107.49$, $P < 0.001$).

The early LAS treatment markedly delayed the appearance of deficits in muscular strength. As shown in Fig. 4, the mutant mice which were treated with LAS from the age of 5 weeks performed better than water-treated FALS mice at 15 and 18 weeks of age (two-way

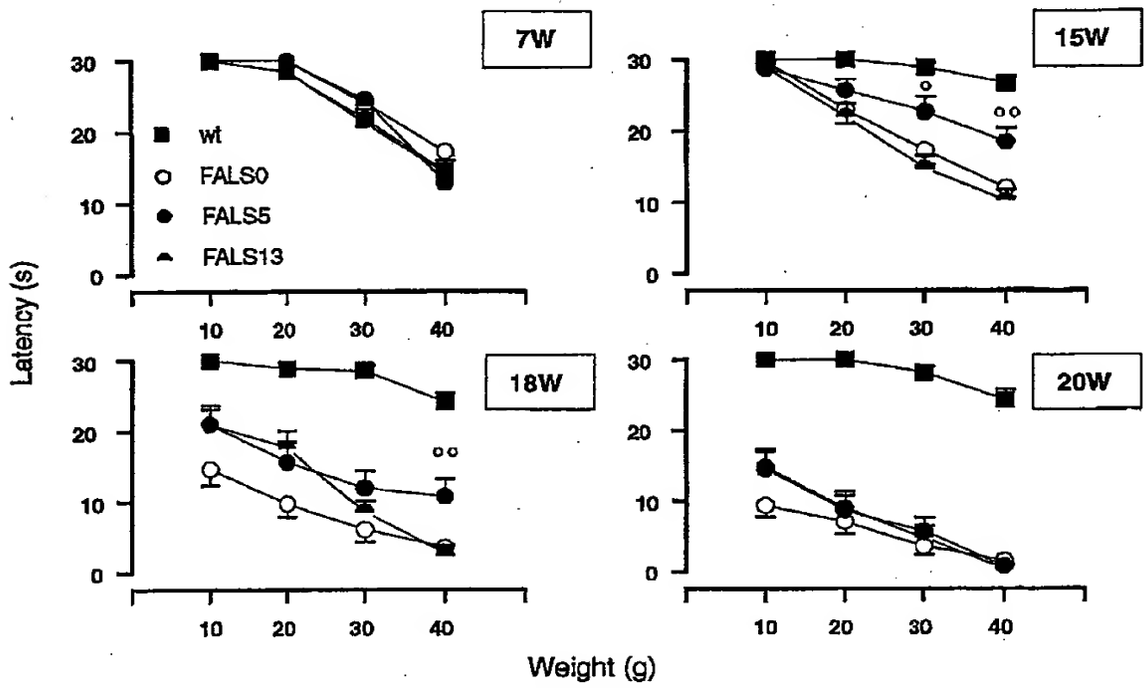

FIG. 4. Muscle strength measured using the loaded grid test at 7, 15, 18, and 20 weeks of age. Note the loss of muscular strength as a function of age in the FALS mice and the beneficial effect of the LAS treatment at 15 and 18 weeks of age. Values are expressed as mean and SEM. See Table 1 for the details of treatment group and for the number of animals per group. Statistical differences between the treated and nontreated FALS mice: oo $P < 0.01$; o $P < 0.05$.

ANOVA weight × group interaction: $F(6,156) = 2.57$, $P = 0.02$; $F(6,156) = 3.54$, $P = 0.003$; respectively). At 20 weeks of age, the two groups were no longer significantly different. The late treatment of LAS did not seem to be beneficial, even if muscle strength had a tendency to be less impaired in the late-treated group than in the water-treated group at 18 weeks of age, as did the reflex from 16 to 18 weeks of age.

Motor coordination (Fig. 5). Motor coordination, estimated by the rotarod test, was also progressively disturbed in FALS mice. As illustrated in Fig. 5, the behavioral performance of the mutant mice decreased from 14 to 21 weeks of age, whereas that of the control wt mice remained stable over the same period (two-way ANOVA: group × age interaction $F(7,203) = 10.46$, $P < 0.001$).

As observed for muscle strength, the early treatment of LAS delayed the appearance of rotarod deficits (two-way ANOVA: group × age interaction $F(14,354) = 3.437$, $P < 0.001$; Fig. 5). The late treatment of LAS had no significant beneficial effect on rotarod performance from 14 to 18 weeks of age, but had a nonsignificant tendency to preserve coordination at 19 weeks of age and after.

Reflex (Fig. 6). The ability of FALS mice to demonstrate the extension of hindlimbs decreased as a function of age: the muscle strength and motor coordination impairments described above were accompanied by a loss of the extension reflex (Fig. 6) starting at 13 weeks of age and which was maximal at 20 weeks of age (two-way ANOVA: group × age interaction $F(14,406) = 31.1$, $P < 0.001$).

The early treatment of LAS delayed the progressive decline of reflex performance [$F(28,728) = 2.411$, $P < 0.001$]. This beneficial effect was more marked than those evaluated on muscle strength and motor coordination, but the decline in performance of treated FALS mice was rapid and sudden after 18 weeks of age.

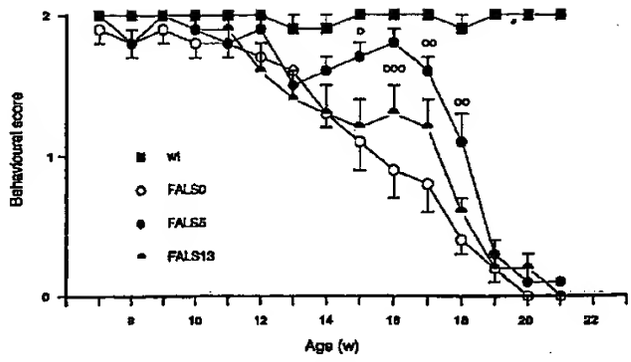

FIG. 6. Evaluation of the extension reflex in the different experimental groups. Note the progressive loss of reflex as a function of age in the FALS mice and the beneficial effect of the LAS treatment. Values are expressed as mean and SEM. See Table 1 for the details of treatment group and for the number of animals per group. Statistical differences between the treated and nontreated FALS mice: ooo $P < 0.001$; oo $P < 0.01$; o $P < 0.05$.

Stride length (Fig. 7). Unlike the behavioral performances described above, impairment of the stride length did not appear until very late, at 20 weeks of age (two-way ANOVA: group × age interaction $F(2,58) = 27.22$, $P < 0.001$). Treatment did not prevent the stride shortening (two-way ANOVA: group × age interaction $F(4,104) = 1.213$, n.s.).

Survival (Fig. 8). In FALS mice, the end-stage criterion was reached at 140–170 days of age and LAS treatment had no beneficial effect on this measure (Log-Rank test, Chi-Square = 3.88, $df = 2$, n.s.).

DISCUSSION

The aim of the present study was to evaluate potential beneficial effects of lysine acetylsalicylate in a

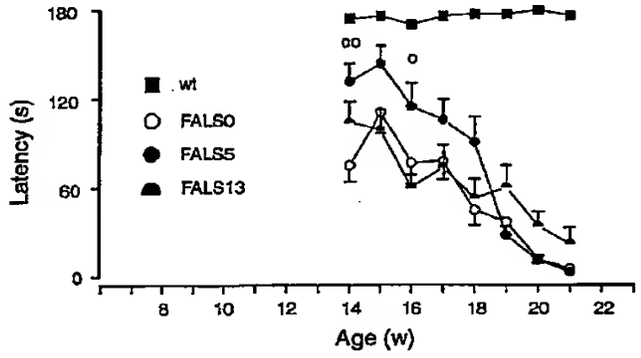

FIG. 5. Motor coordination of experimental groups measured using the rotarod test. Note the appearance of motor incoordination as a function of age in the FALS mice and the beneficial effect of the LAS treatment. Values are expressed as mean and SEM. See Table 1 for the details of treatment group and for the number of animals per group. Statistical differences between the treated and nontreated FALS mice: oo $P < 0.01$; o $P < 0.05$.

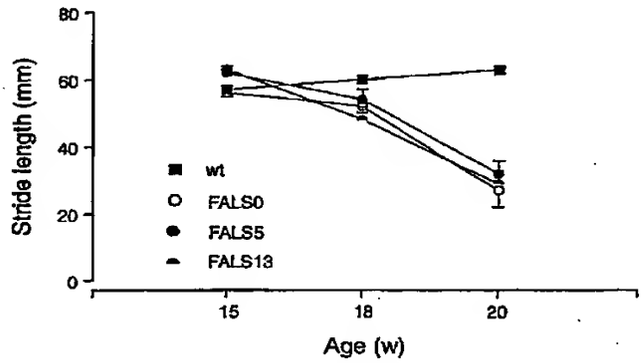

FIG. 7. Stride length of the experimental groups measured with the Medinaceli test. Note the shortening of stride length at 20 weeks of age in the FALS mice and the absence of beneficial effect of LAS treatment. Values are expressed as mean and SEM. See Table 1 for the details of treatment group and for the number of animals per group.

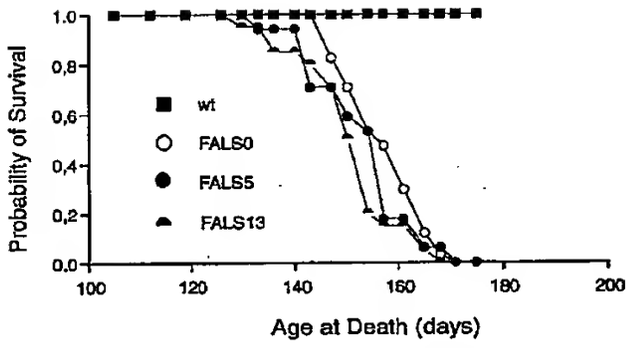

FIG. 8. Survival of FALS mice. Note that LAS treatment has no effect on survival (measured as appearance of end-stage criterion). See Table 1 for the details of treatment group and for the number of animals per group.

model of ALS. Chronic LAS treatment delayed the appearance of motor deficits in FALS mice when the treatment was started early enough. This beneficial effect of treatment was maintained for 6 weeks, until 2 weeks before onset of end-stage criterion. However, LAS treatment had no effect on onset of end-stage disease.

Bioavailability and Tolerance of LAS Treatment

Because mice were administered LAS in drinking water, it was first necessary to evaluate the effect of salicylate on fluid consumption and to verify the ability of salicylate to cross the blood–brain barrier equally in wt and FALS mice. Lysine acetylsalicylate was used in our experiments because of its high solubility in water: immediately after dissolving, LAS is hydrolyzed to aspirin and then to salicylate, which is highly stable in solution. Indeed, we confirmed an unchanged concentration of salicylate in the water bottle up to 1 week after the preparation of the solution (data not shown).

The presence of salicylate and derivatives did not modify fluid consumption of the experimental groups. In addition, the volume of consumed solution (water with or without LAS) remained stable throughout the experiment; i.e., mutant FALS mice exhibiting motor deficits continued to receive the same quantity of aspirin as control wt mice. Our results are in accordance with those of Gurney (23) who reported stable food consumption during aging in mutant FALS mice. The LAS dose consumed by the animals was about 180–300 mg/kg/24 h (aspirin dose: 100–160 mg/kg/24 h), which is lower than the LAS dose of 500 mg/kg/24 h, at which side-effects appear (gastric lesion, body weight decrease, data on file, Synthelabo Research). This is supported by the observation that LAS treatment did not change the evolution of body weight of the mice in our experiment.

Finally, we confirmed the presence of salicylate in the brain of mice after they were administered LAS in drinking water. The amount of salicylate found in the brain was 3 times less than that measured one and a half hours after an i.p. injection of 100 mg/kg aspirin (2). In conclusion, the LAS treatment procedure used in our experiment was suitable for studying the effects of LAS on the progressive motor deficit and on survival in FALS mutant mice.

Progression of Motor Deficits in FALS Mice

The FALS mice have been well characterized in both electrophysiological (3, 28) and histological (13) studies. Motor neuron function starts to become impaired at 7 weeks of age and, in parallel with a reduction in motor unit number, the remaining functional units show an increase in number of neuromuscular junctions compared to controls at 8–9 weeks. The onset of cell death was observed from 13 weeks. We have previously reported some parallels between the time of appearance of these pathologies and the progression of motor deficits in FALS mice (4). Here, we confirmed the motor impairments for certain behavioral measures. For example, extension reflex and motor coordination were impaired from 12 and 14 weeks of age, respectively. Muscle strength of FALS mice, evaluated in the loaded grid task, was also impaired from the age of 13 weeks (results not shown), and in the present studies we evaluated the strength of the animals in more detail. Transgenic mice exhibited mild impairments which depended on the weights of the loaded grid. For example, at 15 weeks of age, FALS mice exhibited poor performance with the heaviest weights, whereas normal performance could be observed for the lightest weight. Finally, we studied the onset of the hindlimb paralysis by measuring the stride length in the Medinaceli task. Unlike the other motor behaviors previously described, paralysis appeared late (after 18 weeks of age) but developed rapidly (50% deficit in 2 weeks). Such a late, but fast, evolution has also been reported by Chiu *et al.* (13) for the same task, although the onset of paralysis was later in our experiment than in those of Chiu (140 and 125 days, respectively). The onset of end-stage criterion reported here was also delayed by about 20 days as compared to that previously observed by us (4) or Chiu *et al.* (13), i.e., 155 days versus 140 and 136 days, respectively. One explanation may be found in a small shift of the G1H genotype. Indeed, the G1H line was identified as a spontaneous expansion in transgene copy number of 40% over the original G1 line described in Gurney *et al.* (25) and thus may be subject to a partial loss of the extra gene copies.

LAS Delays the Appearance of Motor Deficits

The early treatment of LAS delayed the impairment of reflex, muscle strength, and motor coordination in

FALS mice measured by the extension reflex, the loaded grid, and the rotarod, respectively. This motor improvement could be observed up to 18 weeks of age and was particularly marked for the extension reflex. The late treatment was not as efficacious as the early treatment, although there was a tendency for muscle strength and reflex to be less impaired in the late-tested group at 18 weeks of age, or from 16 to 18 weeks of age, respectively. The fact that the treatment which preceded the onset of neuronal dysfunction (i.e., starting at 5 weeks of age) was more efficacious than the one occuring just at the onset of neuronal death (i.e., starting at 13 weeks of age) suggests a neuroprotective effect of LAS. However, the motor performance of treated FALS mice was as poor as that of nontreated mutant mice at 20 weeks of age, and LAS treatment was unable to delay the onset of end-stage disease. The fact that LAS treatment was ineffective on the progression of paralysis was probably because paralysis onset occurred just before death. Indeed, no gait amelioration could be observed at 15 and 18 weeks of age because no clear shortening of stride length could be observed in these ages, and no amelioration was observed at 20 weeks. The early LAS treatment was thus able to delay motor deficits up to two weeks before end-stage disease, which seems to be the maximum effect we can expect for a compound which had no effect on survival.

These results indicate that measures of functional motor progression and survival are separable in the transgenic model and may be affected somewhat independently by therapeutic agents. This idea has already been formulated by Gurney *et al.* (24), who demonstrated differential effects of two compounds in FALS mice: dietary supplementation of the antioxidant vitamin E delayed onset of clinical symptoms but did not prolong survival, whereas riluzole, which interferes with glutamatergic neurotransmission, prolonged survival without any affect on disease onset.

Currently, at least three pathogenic pathways have been proposed to account for ALS: cytoskeletal abnormalities such as excessive accumulation of neurofilaments (15); excitotoxicity resulting from the loss of glutamate transporters (39) and chronic activation of glutamate receptor subtypes (38); and oxidative stress (42). Trophic factors have been the subject of intense interest in recent years with regard to their ability to protect against motor neuron degeneration and CNTF, BDNF, IGF-1, and GDNF have been studied extensively in mouse models of motor neuron degeneration, including the wobbler mouse (34) and the progressive motor neuronopathy (pmn) mouse (40, 41). Although CNTF has been shown to be neuroprotective in the two animal lines, it was completely ineffective in two large clinical trials of ALS patients, probably because of its high toxicity (11, 12). This suggests that, although CNTF was effective in the wobbler and pmn models, the models themselves fail to mimic an important component of the degenerative process in ALS, and thus may not be good as preclinical screening tools. Transgenic FALS mice carrying a SOD1 mutation known to produce the disease in man closely mimic the human pathology and probably represent the best animal model for studying novel therapies for ALS. Indeed, riluzole, which has beneficial effects in humans (7, 32), is also effective in the FALS mouse model. As in humans, CNTF was unable to improve clinical symptoms and survival of transgenic SOD mice. Therefore, compared to other models (mutant mice, facial nucleus lesion) of motor neuron degeneration, the SOD transgenic mouse appears to be a demanding, but perhaps more appropriate, model of ALS for the discovery of promising therapeutic agents such as LAS.

Our results demonstrate that onset of illness (measured by muscular strength, motor coordination, and reflex) was delayed for 2–3 weeks (10–15% of life expectancy) when LAS treatment started at 5 weeks of age. This beneficial effect compares favorably with other results. The overexpression of bcl-2 in FALS mice both delayed onset of illness by 15% and prolonged survival by 12.5% (30), whereas inhibition of interleukine-1β-converting enzyme (ICE) by site-directed mutagenesis prolonged survival by only 8% (20). In the case of pharmacological treatments, riluzole is reported to prolong survival by 8% (24), vitamin E to delay onset of motor neuron disease by 8% (24), and *d*-penicillamine to prolong survival by 10% (27). Comparison between our results and those published by these four teams indicates that the functional improvement in motor performance that we obtained after LAS treatment is among the best thus far demonstrated in this animal model.

Mechanism of Action

The mechanism(s) by which LAS ameliorated motor performance in FALS mice is (are) not yet fully understood. Aspirin is well known for its analgesic, anti-inflammatory, and anticoagulant properties, mediated via inhibition of prostaglandin synthesis (33). Another property of salicylate is its high potency to react with hydroxyl radicals and to be converted to 2,3- and 2,5-dihydroxybenzoic acid (14). We support a neuroprotective role for aspirin, salicylate, and LAS activity in another *in vivo* model of neurodegeneration based on oxidative dysfunction: salicylate, aspirin, and LAS prevent the neurotoxic effects of MPTP in mice (2), a neurotoxin which acts through elevated production of superoxide and peroxynitrite radicals and which is blocked by NO-synthase inhibitors. Wiedau-Pazos *et al.* (42) demonstrated that the SOD mutants A4V and G93A were able to potentiate the oxidative properties of hydrogen peroxide and to induce elevated production of hydroxyl radicals. This enzymatic reaction is copper-

dependent, and a treatment with the copper chelator penicillamine increases survival of FALS mice (27). If motor neuron degeneration in FALS mice is mediated by free radicals, hydroxyl radical scavengers such as aspirin would be expected to act like penicillamine and delay the onset of end-stage disease. We have no explanation as to why LAS failed to delay the onset of end-stage criterion, whereas it considerably delayed the appearance of motor deficits. It is possible that these two antioxidant compounds interact with different cellular compartments or at different levels of the process of neuronal death. Another possibility is that scavenging hydroxyl radicals is not the main mechanism by which aspirin has beneficial effects on motor functions in FALS mice. A recently discovered property of aspirin has been reported by Kwon *et al.* (31), who demonstrated that aspirin inhibits inducible NO synthase and NO production. Moreover, another recent paper (10) questions the presence of elevated concentrations of hydroxyl radicals in the spinal cord of another transgenic FALS strain (43), but suggest instead a role for tyrosine nitration in neurodegenerative events through the peroxynitrite radical. Peroxynitrite radical formation results from the reaction between the superoxide radical O_2^- and the nitrogen monoxide, and SOD mutants have been shown to increase superoxide production in PC12 cells (21). Aspirin may therefore ameliorate motor performance in FALS mice by diminishing the formation of the peroxynitrite radical.

In conclusion, our results demonstrate that the soluble aspirin, i.e., LAS, is able to retard the onset of deficits of coordination of movement, muscle strength, and reflex in this transgenic animal model of ALS. To our knowledge, this functional improvement in motor performance is the most marked thus far demonstrated in this animal model. Whereas we cannot exclude the possibility that the other properties of aspirin may have contributed to the beneficial effect, our results using transgenic SOD mice reinforce the possibility that aspirin may protect from neurodegeneration through antioxidant properties.

There is a clear need for effective treatment of ALS and, to date, riluzole is the only therapeutic compound available. As riluzole seems to prolong survival in humans without affecting onset, cotreatment with a compound which delays onset of motor symptoms, such as aspirin, would be of great value. One of the aims of our work is to communicate to physicians the potential therapeutic usefulness of aspirin, a generic, harmless, and well known drug, in ALS. In particular, early treatment with aspirin (i.e., starting before the onset of symptoms) may be of great benefit in patients with a familial form of ALS.

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Transgenic SOD1 G93A mice develop reduced GLT-1 in spinal cord without alterations in cerebrospinal fluid glutamate levels

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Abstract

Glutamate-induced excitotoxicity is suggested to play a central role in the development of amyotrophic lateral sclerosis (ALS), although it is still unclear whether it represents a primary cause in the cascade leading to motor neurone death. We used western blotting, immunocytochemistry and *in situ* hybridization to examine the expression of GLT-1 in transgenic mice carrying a mutated (G93A) human copper–zinc superoxide dismutase (TgSOD1 G93A), which closely mimic the features of ALS. We observed a progressive decrease in the immunoreactivity of the glial glutamate transporter (GLT-1) in the ventral, but not in the dorsal, horn of lumbar spinal cord. This effect was specifically found in 14- and 18-week-old mice that had motor function impairment, motor neurone loss and reactive astrocytosis. No changes in GLT-1 were observed at 8 weeks of age, before the appearance of clinical symptoms. Decreases in GLT-1 were accompanied by increased glial fibrillary acidic protein (GFAP) levels and no change in the levels of GLAST, another glial glutamate transporter. The glutamate concentration in the cerebrospinal fluid (CSF) of TgSOD1 G93A mice was not modified at any of the time points examined, compared with age-matched controls. These findings indicate that the loss of GLT-1 protein in ALS mice selectively occurs in the areas affected by neurodegeneration and reactive astrocytosis and it is not associated with increases of glutamate levels in CSF. The lack of changes in GLT-1 at the presymptomatic stage suggests that glial glutamate transporter reduction is not a primary event leading to motor neurone loss.

Keywords: excitotoxicity, glial fibrillary acidic protein, motor neurones, superoxide dismutase-1.

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Among the different hypotheses proposed so far for the aetiology of amyotrophic lateral sclerosis (ALS, motor neurone disease), glutamate-mediated excitotoxicity is proposed to play a major role in motor neurone degeneration (Shaw and Ince 1997). Abnormalities of glutamate metabolism, including increased glutamate concentrations in plasma and cerebrospinal fluid (CSF), have been documented in patients with the disease (Rothstein *et al.* 1990), although controversial results have been reported in this respect (Perry *et al.* 1990). More recently a selective loss of the major glutamate re-uptake transporter protein (EAAT2), was found in affected regions of the CNS of ALS patients (Rothstein *et al.* 1995). Decreased glutamate re-uptake into glial cells and/or neurones may result in increased extracellular glutamate levels that could allow excessive stimulation of glutamate receptors on motor neurones and therefore excitotoxic cell death.

However, at present, it is unknown whether alterations in the glutamate transporter and/or increase in the extracellular glutamate levels might be the initial mechanism which leads

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This paper is dedicated to the memory of Dr Rosario Samanin, Head of the Department of Neuroscience of the Mario Negri Institute, who sadly passed away while this manuscript was in preparation (June 4, 2001).

Abbreviations used: ALS, amyotrophic lateral sclerosis; CSF, cerebrospinal fluid; EAAT, excitatory aminoacid transporter; GFAP, glial fibrillary acidic protein; GLT-1 and GLAST, glial glutamate transporters; SOD1, copper–zinc superoxide dismutase.

to motor neurone damage or if these changes are secondary to cell death. These issues cannot easily be addressed from human studies, since examination in the CNS from ALS patients is generally done at the terminal stages of the disease when massive motor neurone loss has already occurred. Transgenic mice overexpressing mutations of copper–zinc superoxide dismutase (SOD1) have been generated, and provide reliable model to investigate the mechanisms underlying motor neurone degeneration. These animals develop progressive limb paralysis associated with spinal motor neurone degeneration, eventually leading to death (Gurney *et al.* 1994; Bruijn *et al.* 1997). It has been shown that the mutant SOD1 protein inactivates the GLT-1 in oocytes upon intra- or extracellular administration of H_2O_2 (Trotti *et al.* 1999). Thus, studies in mouse models of ALS at different stages of the disease progression could be used to determine whether alterations in glutamate transporter activity precede motor neurone degeneration.

Western blotting analysis of the total spinal cord extracts of transgenic mice carrying a G85R mutation of SOD1 suggested a decrease of the main glial glutamate transporter, EAAT-2 (also known in rodents as GLT-1), at the end stage of the disease (Bruijn *et al.* 1997). However, no quantitative analysis was provided in that study, and the study was not able to determine whether the decrease in GLT-1 occurred prior to the onset of the motor neurone pathology or after the disease was fully developed. Furthermore, no anatomical information was provided as to whether changes in glial glutamate transporters occurred in the more vulnerable regions such as the ventral horn of spinal cord. Studies on a different transgenic strain carrying a G93A mutation of SOD1 have shown decreased glutamate uptake into synaptosomes at the end stages of the disease (Canton *et al.* 1998), but since these studies examined neuronal uptake, it is not clear whether they also reflect alterations in glial glutamate transporter levels.

In order to determine whether glutamate abnormalities occur prior to or after the onset of the neuropathological features of ALS, we have used transgenic C57BL/6 mice expressing the human SOD1 with G93A mutation (TgSOD1 G93A). These mice show some morphological alterations of the motor neurones at 8 weeks of age, with an onset of motor symptoms and a significant motor neurone loss starting at 12–14 weeks and progressing to the end-stage symptoms of the disease at about 18–20 weeks (Migheli *et al.* 1999). The time-course of these symptoms differs from other transgenic strains, including TgSOD1G85R. We have used these TgSOD1 G93A mice to examine, at the different stages of the progression of the disease, the levels of glutamate in the plasma and CSF in fasting animals. We have also determined the relative levels of EAAT-2 and its mRNA in spinal cord using immunocytochemistry, *in situ* hybridization and western blotting, comparing the level to glial cell markers, GFAP or EAAT-1(GLAST).

Materials and methods

Materials

All materials were obtained from Sigma (Poole, UK) or Merck (Poole, UK) unless otherwise stated.

Animal model

Transgenic mice originally obtained from Jackson Laboratories and expressing high copy number of mutant human SOD1 with a Gly93-Ala substitution (TgSOD1 G93A) were bred and mantained on a C57BL/6 mice strain at the Consorzio Mario Negri Sud, S. Maria Imbaro (CH), Italy. Identification of transgenic mice was made by PCR (Rosen *et al.* 1993). The mice were maintained at a temperature of 21 ± 1°C with relative humidity 55 ± 10% and 12 h of light. Food (standard pellets) and water were supplied *ad libitum*. For the present study, female mice were killed at 8–10, 14 and 19 weeks of age corresponding, respectively, to a pre-syntomatic, symptomatic and late stage of the progression of the motor dysfunction (see below). Non-transgenic age-matched littermates were used as controls. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. no. 116, G.U. suppl. 40, Feb. 18, 1992, Circolare No. 8 , G.U., 14 luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1 DEC.12, 1987; NIH *Guide for the Care and Use of Laboratory Animals*, US National Research Council, 1996).

Glutamate determination in plasma and CSF

The mice were killed by decapitation after overnight fasting and the blood samples were collected in heparinized tubes and diluted 1 : 1 with 0.5% sodium dodecyl sulphate (SDS) solution, incubated for 10 min at room temperature (20 ± 5°C) and mixed 1 : 1.25 with 10% sulfosalicylic acid solution (v/v). Samples were vigorously mixed and centrifuged. Amino acid analysis was done on 50-μL supernatant fractions using a high performance amino acid analyser (AA Model 6300, Beckman Instrument Inc. Palo Alto, CA, USA), as previously described (Mennini *et al.* 1998). CSF samples were collected from the cisterna magna in Equithesin anaesthetized mice, after overnight fasting, using a glass microcapillary. Samples were rapidly frozen on dry ice. Glutamate was derivatized by mixing the samples with ortho-phthaldialdehyde/β-mercapto-ethanol (1 : 1). After 60 s reaction time the samples were injected onto the column. Peaks were detected using a WATER 470 fluorescence detector and quantified with a Spectra-Physics integrator. Data on glutamate levels in plasma and CSF were analysed by one-way ANOVA followed by Tukey's test for comparison between groups.

Western blot analyses of GLT-1 and GLAST

Mice (seven controls, seven TgSOD1 G93A mice at 10 weeks and six TgSOD1 G93A mice at 19 weeks) were killed by cervical dislocation and spinal cords removed and rapidly frozen. Samples of whole mouse spinal cord from control and transgenic animals were homogenised in protein lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.5 mM phenylmethylsulfonylfluoride, 10 μg/mL leupeptin, 10 μg/mL antipain, 1 μg/mL pepstatin, 1 μg/mL aprotinin, 1 mM Na_3VO_4, 50 mM NaF). Samples were centrifuged at 300 *g* at 4°C for 5 min to remove cell debris and then centrifuged at 35 000 *g* to obtain cytosol and membrane fractions. Protein assays were carried out

using the Bioquant reagent. Membrane (for GLT-1 or GLAST) or cytosolic (for actin) protein samples (20 μg) were mixed 4 : 1 with boiling buffer (50 mM Tris, pH 7.5, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue) and heated to 95°C for 5 min. Samples were separated by electrophoresis on denaturing polyacrylamide gels [8%, acrylamide: bis acrylamide (29 : 1), containing 0.1% SDS], and then electroblotted onto ECL Nitrocellulose membranes (Amersham Pharmacia Biotech, Little Chalfont, UK). Blots were incubated in 4% skimmed milk powder in TBS (20 mM Tris–HCL, 0.5 M NaCl, pH 7.5) for 30 min, washed twice for 5 min in TBS containing 0.05% Tween-20 (TTBS). Blots were then incubated overnight in TTBS containing antisera raised in rabbits against GLT-1 (aB12, 1 : 5000) or GLAST (aA522, 1 : 5000). Both antisera were a kind gift of Dr N. Danbolt (Lehre *et al.* 1995). As a control, mouse anti-actin antibody was used (JLA20, 1 : 1000). The monoclonal antibody developed by J. J. Lin (Lin 1981) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa (Department of Biological Sciences, Iowa City, IA, USA). Membranes were washed three times in TTBS and then incubated for 4 h in peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1 : 5000 in TTBS or peroxidase-conjugated goat anti-mouse IgG (Vector Laboratories, Peterborough, UK). Membranes were washed three times in TTBS, and proteins detected using the ECL plus detection system (Amersham Pharmacia Biotech), according to the manufacturer's instructions. Membranes were exposed to films, which were scanned and the band densities obtained using Bio Image Intelligent Quantifier (B.I. Systems, Ann Arbor, MI, USA). Data were analysed using one-way ANOVA with Tukey's *post hoc* tests as appropriate.

Immunohistochemistry

Mice (three controls for each age group, five TgSOD1 G93A mice at 8 or 18 weeks and three TgSOD1 G93A mice at 14 weeks) were anaesthetized with Equithesin (1% phenobarbitol/4% (v/v) chloral hydrate, 30 μL/10 g, i.p.) and transcardially perfused with 20 mL saline followed by 50-mL 4% paraformaldehyde in phosphate-buffered saline (PBS). Spinal cords were rapidly removed, post-fixed in fixative for 2 h, transferred to 20% sucrose in PBS overnight, then in 30% sucrose/PBS and finally frozen in 2-methylbutane at −45°C. Sections (30 μm) were cut on a cryostat at −20°C through the lumbar spinal cord in the transverse plane at the L_{2-4} level. Sections from each age group were processed at the same time using multiwell plates.

The sections were incubated in 10% normal goat serum in PBS for 1 h and kept overnight in the freshly prepared solution containing antibodies of GLT-1 (guinea pig polyclonal, 1 : 5000, Chemicon International, Temecula, CA, USA) or glial fibrillary acidic protein (GFAP mouse monoclonal, 1 : 250, Boehringher Mannheim). The next day, after washing in PBS, sections were incubated with biotin-conjugated secondary antibody for 1 h, washed and incubated in avidin–biotin–peroxidase (Vectastain kit, Vector Laboratories). After reacting with 3′-3-diaminobenzidine tetrahydrochloride sections were washed, mounted on poly lysine-coated slides, dried, dehydrated through graded alcohols, fixed in xylene and coverslipped using DPX mountant (BDH, Poole, UK) before light microscopic analysis. Control sections were incubated without the primary antibody. The optical density of GLT-1 immunostaining in the ventral and dorsal horn of lumbar spinal cord sections was measured relative to the medial dorsal white matter background of individual sections using an image analyser Imaging System KS300 (Zeiss–Kontron, Munchen, Germany). Optical density was measured within a linear range as determined by increasing dilution of the primary antibody. Ventral horn included the laminae VII, VIII and IX, and dorsal horn included the laminae I,II and III of the grey matter at the L3-L4 level of spinal cord. The optical densities of two sections were quantified for each animal and the mean value of these determinations was used as individual data for statistical analysis by ANOVA one way followed by Tukey's test.

In situ hybridization of GLT-1 mRNA

A rat GLT-1 cDNA fragment was prepared by PCR using standard methods (Molloy *et al.* 1998). A 327-bp fragment corresponding to bases 1310–1636 of the rat sequence (Pines *et al.* 1992) was amplified using specific primers (P3, AGCCGTGGCAGCC ATCTTCATAGC; M9, ATGTCTTCGTGCATTCGGTGTTGGG) and cloned into PCR2.1 (Invitrogen, Carlsbad, CA, USA). Riboprobe template was prepared by amplification of the plasmid (2 ng) in a volume of 500 μL with primers [PCR(T3)-P, ACCGAGCA ATTAACCCTCACTAAAGGGCCGCCAGTGTGCTGGAATTCG; PCR(M13F)-M, CGTTGTAAAACGACGGCC (AG)] that flanked the insert, and incorporated a T3 and T7 site into the template. GLT-1 template was purified using GFX columns (Amersham Pharmacia Biotech, UK).

Antisense and sense cRNA probes were synthesized by *in vitro* transcription from linear DNA templates (1 μg using, respectively, T7 or T3 RNA polymerase enzymes (Promega, Madison, WI, USA) in a reaction mixture containing transcription buffer 1×, dithiothreitol (DTT) 10 μM, RNase inhibitor 30 U (Boehringer Mannheim, Monza, Italy), non-labelled NTP 0,5 mM and UTP 10 μM (Promega) and [^{35}S]UTP 50 mCi (Amersham Pharmacia Biotech). Unincorporated nucleotides were separated from probes using a Quick spin column G50 Sephadex (Boehringer Mannheim), then probes were degraded to 150 base fragments by alkaline hydrolysis. Following ethanol precipitation and denaturation (80°C for 5 min), probes were diluted to 1.5×10^4 cpm/μL with hybridization buffer containing 50% formamide, 2 × sodium saline citrate buffer (SSC), 10 mM Tris–HCl pH 7,5, Denhart's solution 1×, dextran sulfate 10%, 0.2% SDS, 100 mM DTT, 500 μg/mL double-strand Salmon Sperm DNA and 250 mg/mL yeast tRNA.

Spinal cord sections (14 μm) of TgSOD1 G93A mice and controls at different stages of disease progression were cut using a cryostat, and mounted on poly L-lysine-coated microscope slides. The slides were then rapidly immersed in 4% paraformaldehyde freshly prepared in 0.1 M PBS for 5 min, rinsed twice in PBS, acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl pH 8 for 10 min, dehydrated through a graded series of ethanol and delipidated in chloroform. Sections were then air-dried and stored frozen at −70°C until the day of experiment. At the day of the experiment, the slides were brought to room temperature. Then the ^{35}S-radiolabelled riboprobe was applied to each slide and the slides were coverslipped with parafilm and incubated overnight at 42°C.

After hybridization, slides were washed in 2 × SSC at room temperature for 1 h and 2 × SSC at 60°C for 1 h. All slides were

treated with RNase A at 37°C for 30 min (20 μg/mL RNase A in 0.5 M NaCl, 10 mM Tris–HCl pH 7.5, 1 mM EDTA) and washed in 0.1% SSC at 60°C for 1 h. All washing solution contained 10 mM β-mercaptoethanol. Sections were dehydrated through a graded series of ethanols, each containing 0.3 M ammonium acetate, and then air-dried. Slides were exposed to X-ray film (Biomax MR; Kodak, Rochester, NY, USA) for 21 days. Films were developed by X-ray developer, washed and fixed by X-ray fixer (Kodak), then air-dried before optical density quantification using a computerized image analyser KS 300 (Zeiss-Kontron). Optical density was measured within the linear range as determined by using ^{35}S-brain paste standards. For each animals, two spinal cord sections were analysed and the mean of the two values was used for statistical analysis by one-way ANOVA followed by Tukey's test.

Results

Behavioural and histological analysis of mice

The transgenic strain used showed noticeable signs of neuromuscular dysfunction such as limb tremors, deficit in the hindlimbs extension reflex and initial impairment in motor behaviour starting at about 12–14 weeks of age. At 19–20 weeks, all the TgSOD1 G93A mice showed a marked difficulty in the performance of all motor tasks which was associated with paralysis and muscular atrophy of the hind limbs and they died by 142 ± 11 (SD) days. Histologically they show abnormalities in the motor neurones and axons, i.e. vacuolization of the cytoplasm and mitochondria, at about 8 weeks of age, before the symptoms become evident while by 12–14 weeks a progressive loss of motor neurones occurs in the lumbar spinal cord amounting to 44 ± 3% and 52 ± 2% at 12 and 18 weeks, respectively. The number of motor neurones of 8-week-old TgSOD1 G93A mice was unchanged compared with age-matched controls.

For the present study, female mice were killed at 8–10, 14 and 18–19 weeks of age corresponding, respectively, to a presymtomatic, symptomatic and late stage of the progression of the motor dysfunction. Non-transgenic age-matched littermates were used as controls.

GLT-1 and GLAST immunoblotting

Using western blotting to analyse proteins in the size range 35–100 kDa, each of the antibodies recognized a single band of molecular weight 66 kDa for GLT-1, 67 kDa for GLAST and 43 kDa for actin, in line with previously reported data (Lehre *et al.* 1995; Akbar *et al.* 1998) (Fig. 1). The levels of GLT-1 appeared to be decreased in transgenic mice, compared with controls whilst the levels of another glial glutamate transporter, GLAST, were relatively unchanged. Analysis of the data revealed that the levels of GLT-1 tended to be decreased compared with controls, either when the raw data was considered (not shown) or when GLT-1 levels were expressed relative to the amount of actin from the same tissue (Table 1). When compared with level of GLAST in the same samples (Table 1), the level of

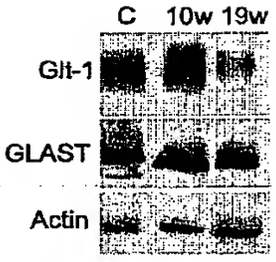

Fig. 1 Representative examples of western blotting of spinal cord protein extracts probed for GLT-1, GLAST and actin. Lanes show samples from control tissue (C), and transgenic mice at 10 and 19 weeks of age. GLT-1, GLAST and actin band sizes were 66 kDa, 67 kDa and 43 Da, respectively. Decreases in GLT-1 in transgenic mice are apparent, without reductions in actin or GLAST.

GLT-1 relative to GLAST was significantly decreased in transgenic mice at 19 weeks, but not 10 weeks, compared with controls (Table 1).

In view of the existence of multimeric complexes of GLT-1 and that oxidative damage, such as might occur during the development of motor neurone disease, might increase the presence of these complexes (Haugeto *et al.* 1996; Trotti *et al.* 1998), samples were analysed in more detail for GLT-1 protein using western blotting. Blots were prepared to analyse bands over a much wider size range (Fig. 2). The results showed that as well as the prominent band of *c.* 66 kDa representing monomeric GLT-1, a larger molecular weight band (*c.* 200 kDa) was present in all the samples, most likely representing a trimeric complex of GLT-1 (Haugeto *et al.* 1996). No bands were found at intermediate molecular weights representing dimeric GLT-1. In addition, for some samples, a lower molecular weight band was found (*c.* 45 kDa), possibly representing a degradation product of GLT-1; the presence or absence of this band did not relate to the disease state.

Quantification of the higher molecular weight (200 kDa) band revealed that there was no increase in TgSOD1 G93A

Table 1 Glutamate transporter levels in spinal cord

	GLT1/actin	GLAST/actin	GLT1/GLAST
Controls	1.54 ± 0.58	1.34 ± 0.41	1.31 ± 0.37
G93A 10-week-old	2.14 ± 0.74	1.22 ± 0.21	2.13 ± 0.78
G93A 19-week-old	0.88 ± 0.29	1.25 ± 0.27	0.70 ± 0.22*

Western blotting was used to determine levels of GLT-1, GLAST and actin in the lumbar spinal cord from control animals ($n = 7$), presymptomatic transgenic animals (G93A 10-week-old, $n = 7$), and end-stage transgenic animals (G93A 19-week-old, $n = 6$). Data were analysed by one-way ANOVA followed by Tukey's test, where appropriate. *$p < 0.05$ compared with controls.

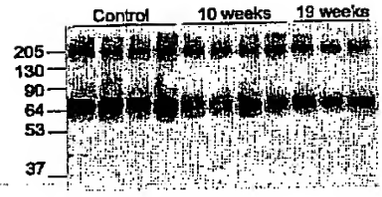

Fig. 2 Western blotting of spinal cord protein extracts probed for GLT-1. Lanes show samples from control tissue and transgenic mice at 10 and 19 weeks of age. GLT-1 immunoreactive bands were found at *c.* 66 kDa, corresponding to monomeric protein and *c.* 200 kDa, corresponding to a multimer and a band of lower molecular weight (*c.* 45 kDa) in some samples. Note that levels of immunoreactivity for the 66-kDa and 200-kDa bands were lower in transgenic mice compared with controls.

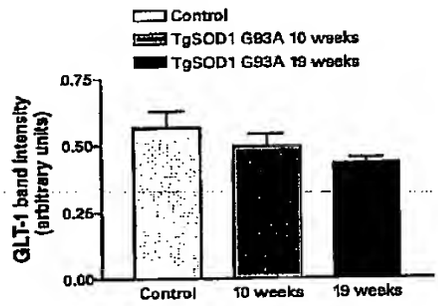

Fig. 3 Quantitative analysis of western blots of spinal cord extracts probed for GLT-1, analysing the intensities of the 200-kDa heteromultimeric band. Band intensities are in arbitrary units. The histograms represent the mean values of four control animals, four TgSOD1 G93A at 10 weeks and three TgSOD1 G93A at 19 weeks.

mice at any of the time points studied; in fact, the levels of the 200 kDa homomultimer were decreased slightly in transgenic mice compared with controls (Fig. 3), as were the levels of GLT-1 monomers.

As the decrease in whole spinal cord GLT-1 was relatively small, pilot experiments of immunoblotting were done separating the dorsal and ventral region of the spinal cord; however, the poor reproducibility of the tissue dissection produced high variability of the samples. Therefore a more detailed analysis of the change in GLT-1 was carried out using immunocytochemistry and *in situ* hybridization, that allowed anatomical resolution of the changes to particular regions of the spinal cord and to glial cells.

GLT-1 immunohistochemistry

In control mice, lumbar spinal cord the GLT-1 immunoreactivity is distributed diffusely throughout the grey matter with the highest immunoreactivity observed in the substantia gelatinosa of the dorsal horn. The white matter shows a filamentous pattern of strong immunostaining in the ventral and lateral regions of the spinal cord while a low signal appears in the dorsomedial region (Fig. 4). This agrees with the distribution described in the rat and human spinal cord, where GLT-1 immunoreactivity has been reported to be exclusively of glial origin (Rothstein *et al.* 1994; Fray *et al.* 1998).

Compared with age-matched controls, a marked, and statistically significant decrease in the GLT-1 immunostaining in the ventral horn of the spinal cord is evident in the TgSOD1 G93A mice at 14 and 18 weeks of age, where motor impairments were present. However, there was no reduction in GLT-1 in presymptomatic animals at 8 weeks of age compared with non-transgenic age-matched mice. No significant changes are observed in the dorsal horn of TgSOD1 G93A mice at any of the ages tested (Fig. 5).

GLT-1 *in situ* hybridization

The riboprobe for GLT-1 revealed widespread distribution of this mRNA thoughout the grey matter of the spinal cord (Fig. 6a), consistent with the expression of this mRNA in astrocytes. Analysis of data showed no change in the levels of GLT-1 mRNA in either the ventral or dorsal regions of the spinal cord of TgSOD1 G93A mice compared with controls (Fig. 6b).

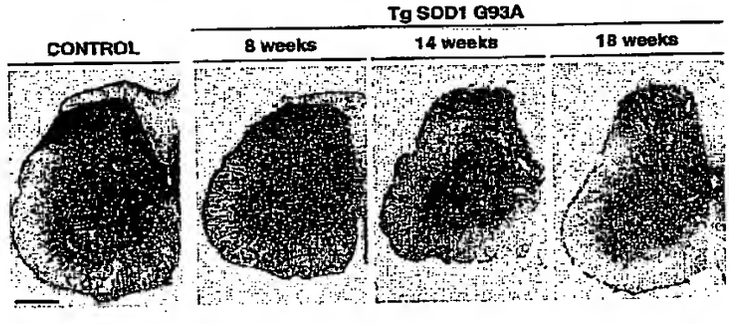

Fig. 4 GLT-1 immunoreactivity in the representative semisections of the lumbar spinal cord of mice. In non-transgenic mice, GLT-1 immunostaining is mainly distributed throughout the grey matter with the highest signal shown at the level of the substantia gelatinosa of dorsal horn. Note a gradual decrease of GLT-1 immunostaining in the ventral and intermediate region of the grey matter in TgSOD1 G93A mice at 14 and 18 weeks of age, while the dorsal horn maintains high immunostaining in TgSOD1 G93A mice at all ages compared with controls. Bar = 250 μm.

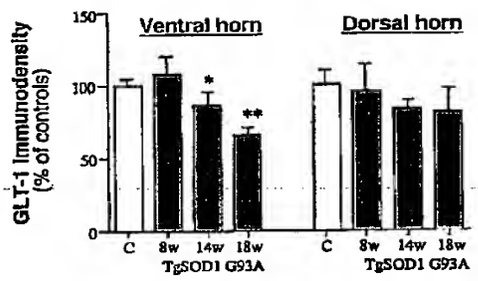

Fig. 5 Quantitative analysis of the GLT-1 immunostaining in the ventral and dorsal horn of the spinal cord. Data are expressed as percent of control. The optical densities of two sections were quantified for each animal ($n = 3–5$) and the mean value of these determinations was used as individual data for statistical analysis by one-way ANOVA followed by Tukey's test. $^{*}p < 0.05$; $^{**}p < 0.01$ compared with controls (C; Tukey's test).

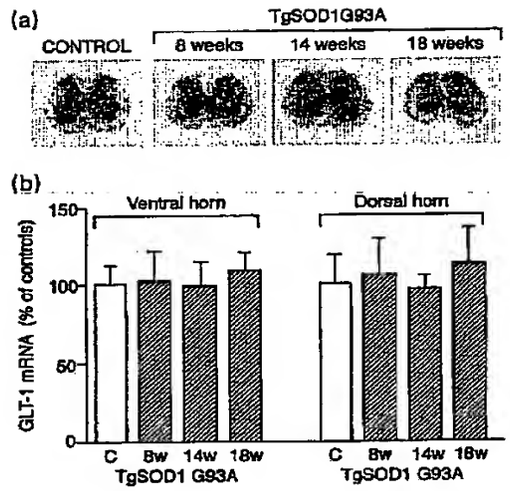

Fig. 6 (a) GLT-1 mRNA in spinal cord determined by *in situ* hybridization. GLT-1 mRNA is found throughout the spinal cord, mainly in the grey matter. There are no clear differences between control and transgenic animals. (b) Quantitative analysis of GLT-1 mRNA in the ventral and dorsal horn of the spinal cord. Data are expressed as percent of control. The optical densities of two sections were quantified for each animal and the mean value of these determinations was used as individual data for statistical analysis by ANOVA one way followed by Tukey's test.

GFAP immunohistochemistry

In the lumbar spinal cord of control mice, the GFAP immunostaining shows a scattered distribution in the grey matter, whereas in the white matter it appears in a filamentous pattern. (Fig. 7). At high magnification, the astrocytes appear with the characteristic stellar shape with a tiny cell body surrounded by branched thin processes (Fig. 7a). In TgSOD1 G93A mice, a progressive increase of GFAP immunolabelling occurs from 8 weeks of age; at the advanced stages (14 and 18 weeks) several astrocytes also show a remarkable hypertrophy of the cell bodies and processes mainly in the ventral region of the spinal cord (Figs 7b–d).

Glutamate levels in CSF and plasma

The mean levels of glutamate in plasma or CSF from all controls were, respectively, 113.2 ± 13.2 and 6.2 ± 0.9 nmol/mL (mean ± SEM). We observed no significant changes in the CSF glutamate concentrations of TgSOD1 G93A mice at 14 and 18 weeks of age in respect to age-matched control mice (Fig. 8). No significant differences were also observed in the plasma levels of glutamate in TgSOD1 G93A mice at 8 and 14 weeks compared with age-matched controls, whereas at 18 weeks the levels were significantly lower (Fig. 8).

Discussion

In this study we have carried out a detailed characterization of the time-course of changes in glutamate, and the main glutamate transporter protein GLT-1 (EAAT-2), in SOD1 G93A transgenic mice. We have focussed on the spinal cord, where the main loss of motor neurones is found. Our findings reveal that there are changes in glial cell markers: the glutamate transporter GLT-1 is decreased, the glial cytoskeletal marker, GFAP, is increased and GLAST is unchanged. The decrease in glutamate transporter levels do not result in a measurable change in CSF glutamate level. The biggest changes are in the most vulnerable regions of spinal cord, and are found at end stages of the disease.

Time course and regional selectivity of GLT-1 reduction

The present study reveals that levels of the protein GLT-1 (EAAT-2), but not its mRNA, are significantly decreased in the ventral grey region of lumbar spinal cord of TgSOD1 G93A mice at the advanced, but not at the presymptomatic, stage of the disease. Using immunocytochemistry and western blotting, we found changes in GLT-1 in transgenic animals in the whole spinal cord at 19 weeks, and changes in GLT-1 in the ventral horn of the spinal cord at 14 and 18 weeks. At these time points, there is marked motor neurone death and clear symptoms of the disease. These results are consistent with previous observations on a similar transgenic strain, which showed that synaptosomal glutamate uptake was not decreased between 8 and 17 weeks in transgenic mice, but was significantly reduced at 21 weeks (Canton *et al.* 1998). The present results suggest that alterations in glial expression of glutamate transporter expression occur, but only at the late stages of the disease.

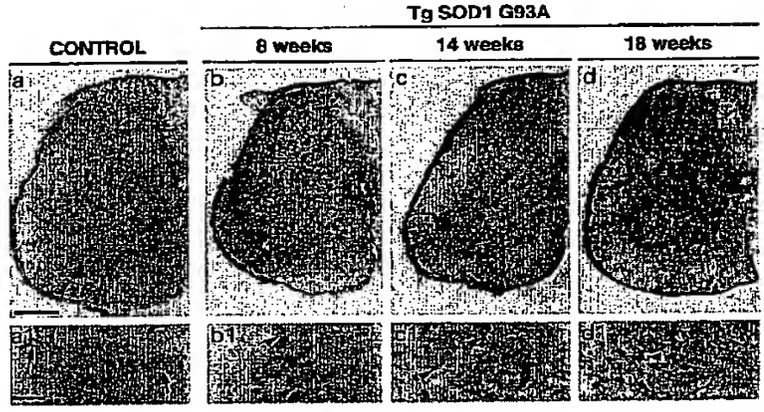

Fig. 7 GFAP immunoreactivity in the lumbar spinal cord of mice. In non-transgenic mice, GFAP immunostaining is scattered distributed in the grey matter with a filamentous pattern in the white matter. In (a–d) note a gradual increase of the GFAP immunostaining in the whole section of the spinal cord in TgSOD1 G93A mice at the different ages with a prominent increase in the grey matter of 14- and 18-week-old mice. (a1–d1) High magnification of the astrocytes from the ventral horn region shows in control mice a characteristic stellar shape with a tiny cell body surrounded by branched thin processes (arrows), whereas in TgSOD1 G93A mice, the astrocytes appeared more intensely stained with hypertrophic cell bodies and processes, particularly in 18-week-old mice. Bar = 250 μm (a–d) and 50 μm (a1–b1).

At 8 weeks of age these transgenic mice do not show motor neurone loss or clinical symptoms although morphological alterations of motor neurones are apparent, for example widespread vacuolization of cytoplasm and swelling of mitochondria (Migheli *et al.* 1999; Bendotti *et al.* 2001). As reported here, initial signs of reactive astrocytosis, as demonstrated by enhanced immunolabelling for GFAP, are also found at this age. Therefore, the present results suggest that the early stage of motor neurone damage in TgSOD1 G93A mice is not associated with changes in GLT-1 protein levels.

Increasing evidence indicates that glutamate transporters, and in particular GLT-1, are vulnerable to the oxidation resulting in an impaired glutamate uptake function (Trotti *et al.* 1998). In particular, it has been shown that the mutant SOD1 protein inactivates the GLT-1 in oocytes upon intra- or extracellular administration of H_2O_2 (Trotti *et al.* 1999). Thus, although our study demonstrates that the level of GLT-1 is unchanged in the lumbar spinal cord of young transgenic mice, we cannot exclude that the activity of this transporter is already compromised at this age. This may play a role in the initial process of motor neuronal death by mediating an increase in extracellular glutamate concentration. Interestingly, recent studies (Alexander *et al.* 2000; Andreassen *et al.* 2001) showed, by intracerebral microdialysis, an increase of extracellular fluid glutamate in somatosensory cortex of TgSOD1 G93A mice, at the advanced stage of the disease, without visible pathology in this brain region. This effect was magnified by the perfusion with a glutamate uptake inhibitor or by the NMDA glutamate receptor stimulation, whereas it was significantly attenuated by creatine treatment which also increased survival and motor performance of TgSOD1 G93A mice (Andreassen *et al.* 2001). This effect was interpreted as a decreased capacity to clear glutamate from the extracellular space although the cortical GLT-1 levels measured by immunoblotting appeared unchanged (Alexander *et al.* 2000). It has been reported that oxidative conditions may induce the formation of GLT-1 homomultimers, thus reducing the amount of monomer revealed by immunoblotting (Haugeto *et al.* 1996), and evidence suggests that oligomeric structure of GLT-1 is required for transport activity. Although there is substantial evidence for oxidative damage in transgenic mice with G93A SOD1 mutation (Andrus *et al.* 1998), we did not observe an increase but rather a slight decrease in the homomultimeric form in these mice at the advanced stage of the disease. This indicates that oxidative damage which might lead to the inactivation of

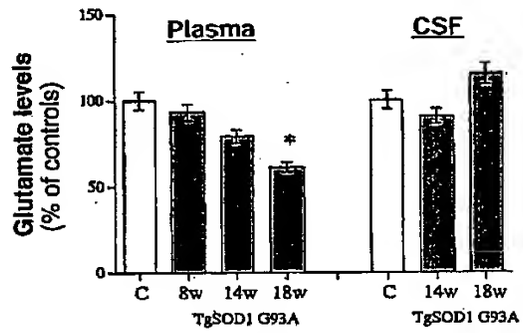

Fig. 8 Glutamate in CSF and plasma from transgenic mice. Concentrations of glutamate in plasma and cerebrospinal fluid (CSF) from TgSOD1 G93A mice at different ages compared with controls (C; white bars). Values are expressed in percent of age-matched non-transgenic controls. Each bar represents the mean ± SE. *$p < 0.05$ compared with respective controls (one-way ANOVA).

GLT-1 in TgSOD1 G93A mice at the early stage, might, in the long-term, reduce the transporter levels.

Our study demonstrates that the decrease of GLT-1 specifically occurs in the regions affected by the pathology since no significant changes in immunoreactivity were observed in the dorsal horn of the spinal cord where there is no neuronal degeneration. Our results are in line with those obtained from post-mortem tissues of patients with sporadic and familial ALS showing a selective decrease of GLT-1 in spinal cord and motor cortex but not in other unaffected brain regions (Rothstein *et al.* 1995). More recently, Fray *et al.* (1998) using quantitative EAAT2 immunohistochemistry, have shown a decrease in all regions of the lumbar spinal cords from motor neurone disease patients, although the highest reduction was observed at the level of the ventral horn and intermediate grey, the most affected areas. Moreover, as found in ALS patients (Bristol and Rothstein 1996), we have not found changes in the levels of mRNA of GLT-1 in the lumbar spinal cord of SOD1 G93A at any stage of the disease. Thus, these results suggest that the decrease of GLT-1 is due to a post-transcriptional regulation of this protein.

Alterations in GLT-1 compared with other glial proteins, GLAST and GFAP

The changes in GLT-1 seem to be specifically associated with disease progression, compared with the other glutamate transporter studied. We observed by quantitative immunoblotting a decrease of the GLT-1 but not GLAST in the lumbar spinal cord extract of SOD1 G93A mice during the progression of the disease, suggesting a differential regulation of these two glial glutamate transporters under these pathological conditions. Since it has been reported that the expression of glutamate transporter subtypes in astrocytes is under neuronal regulation (Gegelashvili *et al.* 1997; Swanson *et al.* 1997; Perego *et al.* 2000), we suggest that the decrease in GLT-1 observed in the transgenic FALS mice, as well as in the post-mortem tissue of patients with ALS, results from a loss of neuronal factors regulating the expression of this glial glutamate transporter in the vulnerable regions. The fact that GLAST was not changed suggests that these two glial transporters in the spinal cord may be regulated differently by neuronal factors. This is in line with studies in cultures of cortical astrocytes that showed that the expression of GLT-1 is much more dependent on neuronal factors and intact neurones compared with GLAST (Gegelashvili *et al.* 1997).

The loss of GLT-1 occurred in the spinal cord of motor impaired mice in the presence of strongly activated astrocytes in this region as demonstrated by their hypertrophy and increased immunostaining of GFAP. This agrees with a previous study of Mennini *et al.* (1998) showing, by immunoblotting, a significant decrease of GLT-1 associated with an increase in GFAP in the spinal cord of *mnd* mice, a strain which develops neuronal ceroid lipofuscinosis with a late-onset paralysis of hind limbs (Bronson *et al.* 1993). Moreover, a down-regulation of GLT-1 together with an increase in GFAP was reported in rat brain following lesion of corticostriatal pathway (Levy *et al.* 1995). This suggests that hypertrophic astrocytes show an altered functional activity which may induce a down-regulation of the GLT-1, but not GLAST, glutamate transporter.

Glutamate transporters and CSF glutamate levels

In this study, we did not find any increase in the levels of glutamate in the CSF of transgenic mice. Our findings differ from observations in ALS patients which showed increased CSF levels of glutamate (Rothstein *et al.* 1990). Nevertheless, controversial evidence exists in this respect (Perry *et al.* 1990).

CSF concentration of glutamate reflects multiple processes including release and uptake, as well as glutamate metabolism. In this regard, decreased activity of glutamate dehydrogenase (GDH), an enzyme playing a central role in glutamate metabolism, has been suggested to be responsible of the increased levels of glutamate in the CSF and plasma of ALS patients (Plaitakis 1990), although a down-regulation of EAAT2 may also contribute to this effect. The lack of increase in the levels of glutamate in the CSF and plasma of transgenic mice therefore suggests that the GLT-1 reduction observed in the ventral spinal cord of these mice does not significantly modify the total pool of extracellular glutamate. We cannot exclude the possibility that an increase in the extracellular concentration of this amino acid, with functional relevance for the neurodegenerative process, may occur in the vicinity of motor neurones in the ventral spinal cord of TgSOD1 G93A mice as consequence of GLT-1 reduction.

The decrease in plasma glutamate levels observed at the late stage of the disease is likely to be consequent to muscle loss in these mice reducing the pool of metabolic glutamate. A similar decrease in plasma glutamate levels has been found in *mnd* mice at the advanced stage of the disease, although at the stage preceding the onset of neuromuscular deficit they showed increased plasma concentration of glutamate (Mennini *et al.* 1998).

Conclusion

In conclusion, this study clearly demonstrates a selective GLT-1 decrease in the spinal cord of TgSOD1 G93A mice which is likely due to a loss of neuronal factors following motor neurone death and/or to a sustained long-lasting oxidative damage of the protein. This effect therefore is not a primary event leading to motor neurone degeneration but may contribute to the rapid progression of the disease at the advanced stage.

Acknowledgements

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Neurodegeneration Is Associated to Changes in Serum Insulin-like Growth Factors

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Serum levels of insulin and insulin-like growth factors and their binding proteins (IGFs and IGFBPs, respectively) are changed in human neurodegenerative diseases of very different etiology, such as Alzheimer's disease, amyotrophic lateral sclerosis, or cerebellar ataxia. However, the significance of these endocrine disturbances is not clear. We now report that in two very different inherited neurodegenerative conditions, ataxia-telangiectasia (AT) and Charcot-Marie-Tooth 1A (CMT-1A) disease, serum levels of IGFs are also altered. Both types of patients have increased serum IGF-I and IGFBP-2 levels, and decreased serum IGFBP-1 levels, while only AT patients have high serum insulin levels. Furthermore, serum IGFs are also changed in three different animal models of neurodegeneration: neurotoxin-induced motor discoordination, diabetic neuropathy, and hereditary cerebellar ataxia. In these three models, serum insulin levels are significantly decreased, serum IGF-I and IGFBP-1, -2, and -3 are decreased in diabetic and neurotoxin-injected rats, while serum IGFBP-1 is increased in hereditary ataxic rats. Altogether, these observations indicate that a great variety of neurodegenerative diseases show endocrine perturbations, resulting in changes in serum IGFs levels. These perturbations are disease-specific and are probably due to metabolic and endocrine derangements, nerve cell death, and sickness-related disturbances associated to the neurodegenerative process. Our observations strongly support the need to evaluate serum IGFs in other neurodegenerative conditions. © 2000 Academic Press

Key Words: **insulin-like growth factors; neurodegeneration; ataxia; diabetes; Charcot-Marie-Tooth disease.**

INTRODUCTION

Recent progress in human genetics have revealed the existence of specific mutations in many types of neurodegenerative diseases (Hardy and Gwinn-Hardy, 1998). Because the affected proteins are usually widely expressed in the nervous system, the relationship between changes in protein function and appearance of specific patterns of cell death is not yet well understood (Price *et al.*, 1998). Conceivably, the primary pathogenic effect will lead to death of those cells directly affected by the mutation. Subsequent homeostatic derangements associated to this primary cell death may include a loss of appropriate trophic input to nerve cells not primarily affected by the disease. As a consequence, secondary cell death will proceed. A similar general process is envisaged for nongenetic neurodegenerative processes such as those taking place after isquemic insult, physical injury, or neurotoxin-related neuronal death. Thus, changes in growth factor input, among other pathological alterations, may be an additional factor involved in the progression of neurodegenerative processes. In this regard, we and others previously reported altered levels of

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insulin, insulin-like growth factor I (IGF-I), and IGF-binding proteins (IGFBPs) in different human neurodegenerative diseases (Tham *et al.*, 1993; Torres-Aleman *et al.*, 1996, 1998; Schwab *et al.*, 1997; Craft *et al.*, 1998). More recently, we also found that insulin-like growth factor I (IGF-I) is involved in the progression of neuronal death after neurotoxin insult in rats (Fernandez *et al.*, 1999).

The insulin family of growth factors include insulin, IGF-I and -II, and the IGFBPs. The latter are proteins that modulate the biological activity of the IGFs (Jones and Clemmons, 1995). These peptides are found at high levels in the blood stream, but are also present in the developing and adult nervous system where they exert wide trophic actions (Torres-Aleman, 1999). IGF-I has recently been found of potential therapeutical use in different neurodegenerative conditions (Fernandez *et al.*, 1998; Pulford *et al.*, 1999). The rationale for these studies is based on the potent trophic actions of IGF-I and on its ability to rescue injured neurons after a great diversity of insults (Dore *et al.*, 1997; Torres-Aleman and Fernandez, 1998). The observation of low serum IGF-I levels in human patients suffering from different types of neurodegenerative diseases and in animal models (Torres-Aleman *et al.*, 1996; Busiguina *et al.*, 1996; Schwab *et al.*, 1997; Scheepens *et al.*, 1999) gave support to this therapeutic approach. However, it is still unclear whether insulin (Wickelgren, 1998), IGF-I (Torres-Aleman *et al.*, 1996, 1998; Dore *et al.*, 1997), or for that matter any neurotrophic factor, are directly involved in the progression of the neurodegenerative process. In the present study we have evaluated widely different types of etiopathogenic processess involved in neurodegeneration to determine whether changes in serum levels of these growth factors are characteristic of a subset of neurodegenerative diseases or, on the contrary, they changed in all types of neurodegenerative conditions regardless of their origin. We include both human diseases and animal models in our study to have a broader sample of neurodegenerative mechanisms. Specifically, we have explored whether both acute (neurotoxic insult) or slow (metabolic derangement) nongenetic neurodegenerative processes as well as hereditary neurodegenerative diseases (ataxia-telangiectasia, Charcot-Marie-Tooth 1A disease, and cerebellar atrophy) will present changes in these trophic factors. We found significant changes in serum insulin, IGF-I, and IGFBPs levels in all these neurodegenerative conditions of widely different phenotypes or genotypes.

MATERIALS AND METHODS

Patients

The series comprises 29 patients with an inherited peripheral neuropathy of the Charcot-Marie-Tooth 1A (CMT-1A) disease type. Most of these patients belong to six previously reported families (Hallan *et al.*, 1992; Garcia *et al.*, 1998). Mean age was 33.4 years, with a range of 8–77 years. All patients showed slight to moderate signs of peroneal muscular atrophy, marked and uniform slowing of nerve conduction velocity, and a tandem duplication of a 1.5-Mb region in chromosome 17p11.2. The weight/height ratio of CMT-1A patients was within normal population parameters. A second series of 12 patients with an inherited central neurodegeneration, ataxia-telangiectasia (AT), were also studied (13.2 years; range, 3–34). They were genotyped, diagnosed, and their blood samples collected through a multicenter program organized by the AT Children's Project (Boca Raton, FL). Age-matched normal subjects (age range of 6–68 years) were used as controls: 10 for CMT-1A (34.4 years) and 8 for AT (12.2 years). Since the two populations of control subjects showed similar values for serum IGF-I and insulin they were pooled and shown as a single control population. Blood samples were withdrawn in fasted conditions between 8 and 10:00 a.m.

Animal Models

Neurotoxin-induced cerebellar ataxia. Cerebellar deafferentation resulting in ataxia was induced in adult rats (250–300 g) by injection of the neurotoxin 3-acetylpyridine (3AP, 50 mg/kg ip) as described in detail before (Fernandez *et al.*, 1997). Massive neuronal loss (99%, $P < 0.001$) was found specifically in the inferior olive (Torres-Aleman *et al.*, 1991). Animals show permanent and severe ataxia as measured in the rota-rod test (Fernandez *et al.*, 1998). They also had a 10% decrease in body weight as compared to controls.

Hereditary cerebellar ataxia. Two to three month old "shaker" mutant rats with hereditary ataxia due to progressive primary Purkinje cell death were used (Tolbert *et al.*, 1995). At these ages most Purkinje cells in the cerebellar anterior lobe and many of these cells in the posterior lobe have degenerated. Coincident with this loss of Purkinje cells the mutant rats are ataxic characterized by a wide-based "staggering" gait. Ataxic animals weighed 20–28% less than age-matched controls (Wolf *et al.*, 1996).

Diabetic neuropathy. Insulin-dependent diabetes was induced in adult rats by injection of the pancreatic beta-cell toxin streptozotocin (65 mg/kg, ip) as described (Busiguina *et al.*, 1996). Diabetic rats had significantly high serum glucose levels (396 ± 16 mg/dl as compared to 72 ± 2 mg/dl in controls) and developed a peripheral neuropathy as determined by loss in the paws of the sensitivity to heat ($P < 0.05$ as compared to controls). Central-neuronal loss (Biessels *et al.*, 1994) was ascertained by counting cerebellar Purkinje cells; we found a 25% reduction in the number of this type of neurons ($P < 0.05$) after 2 months of induction of insulin-dependent diabetes. Animals show also a severe body mass loss (50% of controls).

Immunoassays

All assays used have been described in detail before (Busiguina *et al.*, 1996; Torres-Aleman *et al.*, 1996, 1998). Two types of samples were analyzed: serum from human subjects and from experimental animals; and cerebellar tissue from experimental animals. We chose the latter as a representative brain area because previous findings indicated that the cerebellum shows specific changes in the levels of insulin-like growth factors in neurodegenerative diseases (Torres-Aleman, 1991, 1996). IGF-I, IGFBPs, and insulin levels were measured by either radioimmunoassay (IGF-I, insulin, IGFBP-2, and BP-3), ELISA (BP-1), or Western ligand blot (WLB). The latter technique was used to measure intact BP-3 in human sera as determined by its affinity to bind labeled IGF-I. Immunoreactive BP-3 levels in human serum do not reflect intact levels of BP-3 because most anti-human anti-BP-3 antibodies recognize BP-3 fragments (Marks *et al.*, 1991). WLB was used also to measure rat IGFBPs in serum and brain tissue. In all cases, control and experimental samples were alternated in the blot to minimize intrablot variations. WLB results were analyzed by laser densitometry and results express as percentage of control levels within the same blot (see figures).

Serum samples were either directly measured (IGFBPs and insulin) or extracted (for IGF-I) using Sep-pak cartridges as reported (Pons and Torres-Aleman, 1992). Brain tissue samples were processed for IGF-I RIA or BP-2 WLB as described (Pons and Torres-Aleman, 1992; Busiguina *et al.*, 1996). Results are shown as mean SEM. A Student's *t* test was used for statistical analysis. A value of $P < 0.05$ was taken as significantly different.

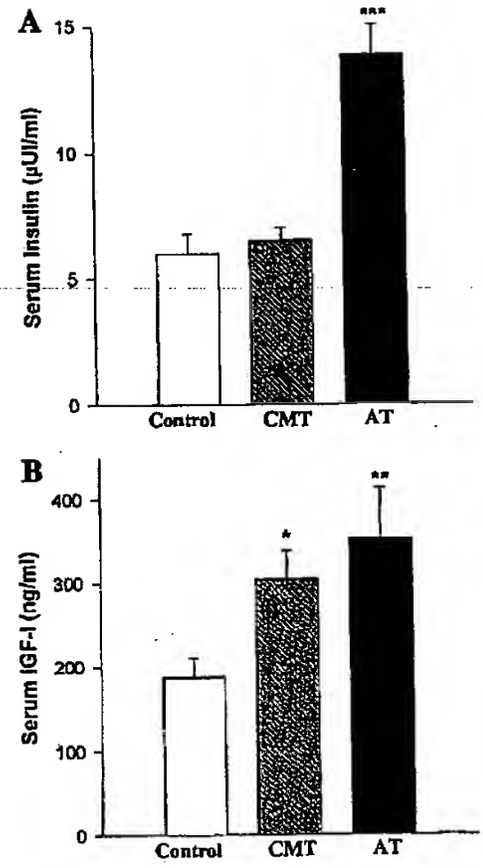

FIG. 1. Levels of insulin-like growth factors in human inherited neurodegeneration. Serum insulin (A) and IGF-I (B) levels in Charcot–Marie–Tooth 1A disease (CMT) patients, ataxia-telangiectasia (AT) patients, and control subjects. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0001$.

RESULTS

Serum Insulin-like Growth Factors in Hereditary Neurodegenerative Diseases

We previously reported that in genetically heterogenous cerebellar ataxic patients IGF-I and insulin levels are significantly depressed (Torres-Aleman *et al.*, 1996). We now studied two human neurodegenerative conditions where the genetic perturbation is homogenous and identified. Ataxia-telangiectasia (AT) is associated to a mutation in Atm, a protein belonging to the superfamily of PI3-kinases (Savitsky *et al.*, 1995), while Charcot-Marie-Tooth type 1A disease (CMT-1A) is linked to a duplicaton of the gene for PMP22, a constitutive myelin protein (Hanemann and Muller, 1998). As shown in Fig. 1, serum IGF-I levels were significantly increased in both types of patients while

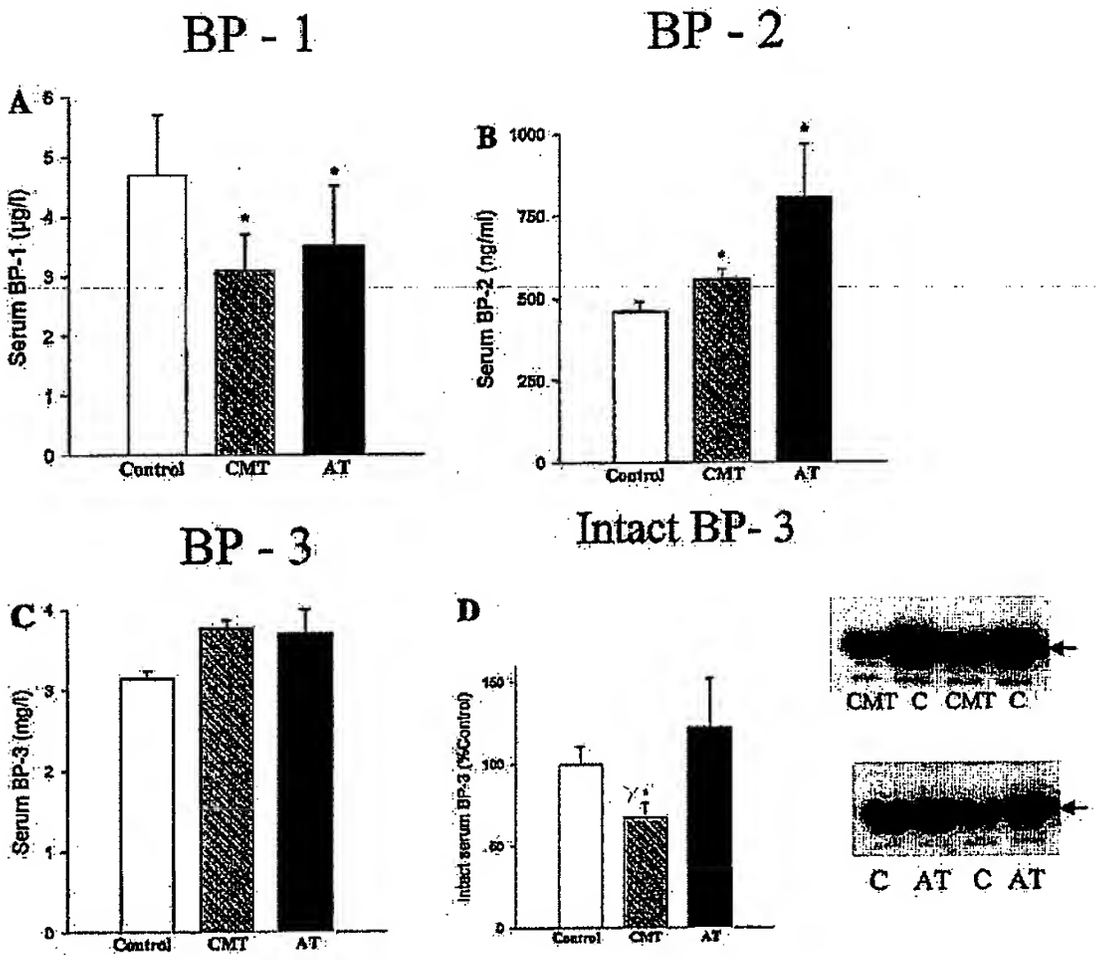

FIG. 2. Serum levels of BP-1 (A), BP-2 (B), and BP-3 (C) in CMT and AT patients and in controls (c). Intact BP-3 (D) determined by its affinity to bind IGF-I (WLB) was significantly changed in CMT patients. Representative WLBs of BP-3 in CMT and AT patients and controls are shown to the right in D. *$P < 0.05$.

serum insulin levels were significantly increased only in A-T patients. Similarly, serum levels of 2 of the 3 major types of circulating IGFBPs were also altered (Fig. 2). Although immunoreactive BP-3 levels were not changed (Fig. 2C), bioactive levels, as determined by the affinity of BP-3 to bind IGF-I, were slightly increased in AT patients (122 ± 35% of control values) and significantly decreased in CMT patients (67 ± 9% of controls, $P < 0.05$; Fig. 2D).

We then extended these observations to an animal model of cerebellar ataxia (the "shaker" rat) due to X-linked inheritance of the shaker genotype which results in the adult-onset degeneration of Purkinje cells. These rats have low serum insulin levels, and although they also have very low cerebellar IGF-I levels, serum IGF-I levels are normal (Fig. 3). In addition, serum and cerebellar IGFBPs are also modified in these animals (Fig. 3).

Serum Insulin-like Growth Factors in Nongenetic Neurodegeneration

Altered serum insulin and IGF-I levels have also been found in human and experimental neurodegenerative conditions not linked to genetic mutations (Tham *et al.*, 1993; Schwab *et al.*, 1997; Craft *et al.*, 1998; Fernandez *et al.*, 1998; Scheepens *et al.*, 1999). We now have extended these observations in two different models of experimental neurodegeneration. A first one consists of an acute neurodegenerative process due to toxic insult (3AP). In this model, neuronal death is circumscribed to the first week after neuro-

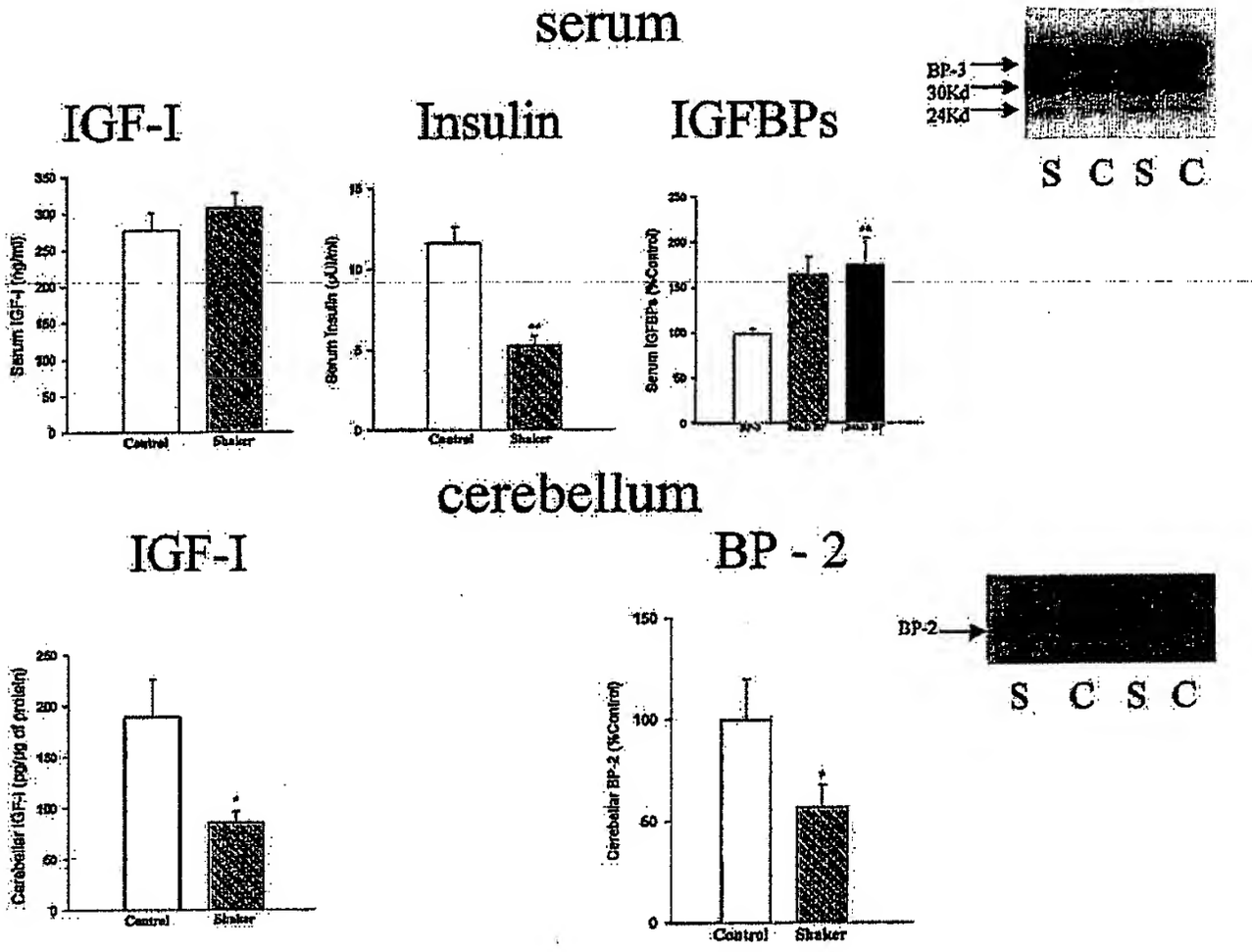

FIG. 3. Insulin-like growth factors in an animal model of hereditary cerebellar ataxia (shaker rat). Upper panel: serum levels of insulin, IGF-I, and IGFBPs in shaker ataxic rats. Lower panel: cerebellar levels of IGF-I and BP-2 in shaker rats. Upper inset shows a representative WLB for serum IGFBPs while the lower inset shows a representative WLB for BP-2 in the cerebellum. S, shaker rat; C, control rat. Arrows in gels indicate position of the different IGFBPs. $^{*}P < 0.05$; $^{**}P < 0.001$.

toxin administration (Fernandez *et al.*, 1999). A second one is a prolonged neurodegenerative process due to diabetes-related metabolic derangements, where cell death develops gradually and continues unabated. As previously reported (Busiguina *et al.*, 1996; Fernandez *et al.*, 1998), both types of insults produce low serum IGF-I levels. However, time-course analysis of the changes indicate that in diabetic animals serum IGF-I remains low for the duration of the study (8 weeks), while 3AP-injected rats show a recovery of serum IGF-I 4 weeks after injection of the neurotoxin (Fig. 4A). If the components of the IGF trophic system are evaluated at a time when serum IGF-I levels are depressed, i.e., 2 weeks after 3AP and 8 weeks after streptozotocin, most of them are also altered. Thus, as shown in Fig. 4B, serum insulin is significantly decreased. Similarly, a pronounced decrease in cerebellar IGF-I levels is also found (Fig. 4C). Finally, both serum and cerebellar IGFBPs are also low (Figs. 4D and 4E). However, if these components are evaluated 4 weeks after 3AP injection, when IGF-I levels are back to normal, all of them are also normalized (not shown).

DISCUSSION

The present findings extend and reinforce the observation that levels of circulating insulin, IGF-I and IGFBPs are altered in many types of human neurode-

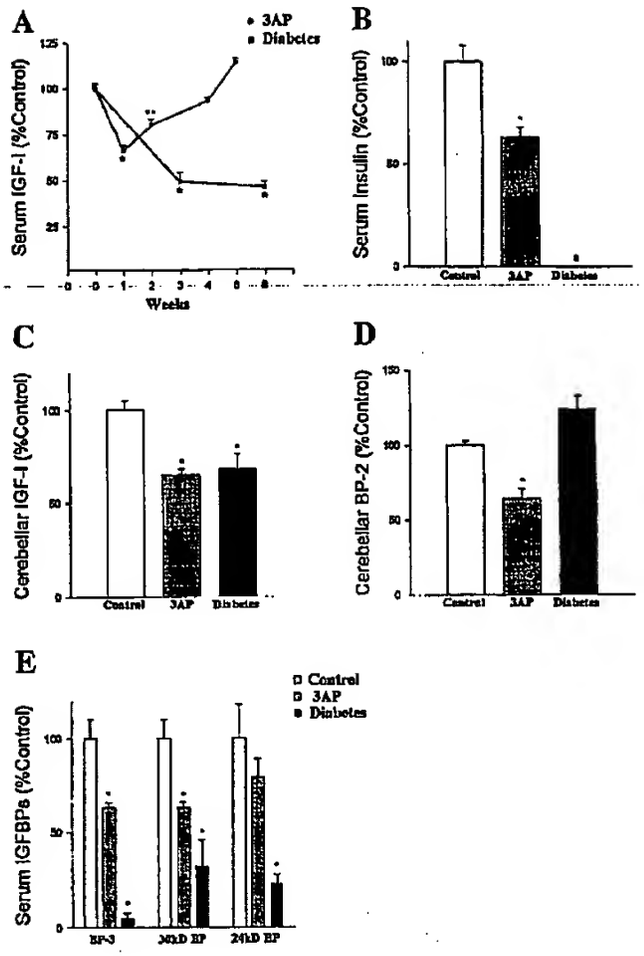

FIG. 4. (A) Time course analysis of serum IGF-I levels in acute, neurotoxin-induced (3AP) neurodegeneration, and in long-term neurodegeneration (diabetes). (B) Serum levels of insulin 2 weeks (3AP) and 8 weeks (diabetes) after toxin administration. (C) Cerebellar levels of IGF-I in ataxic (3AP) and diabetic animals. (D) Cerebellar levels of BP-2 in 3AP-treated and diabetic animals. (E) Serum IGFBPs in 3AP-induced ataxic rats (3AP) and in insulin-dependent diabetic rats. a, undetectable levels. $^{*}P < 0.01$; $^{**}P < 0.05$.

generative diseases, including major illnesses such as Alzheimer's disease or stroke as well as inherited neurodegenerative diseases (Tham *et al.*, 1993; Torres-Aleman *et al.*, 1996, 1998; Schwab *et al.*, 1997; Craft *et al.*, 1998). Similarly, changes in serum insulin, IGF-I and IGFBPs are found in various animal models of neurodegeneration (Torres-Aleman *et al.*, 1991; Fernandez *et al.*, 1998; Zhang *et al.*, 1997, 1999). Although experimental models do not mimic all aspects of human disease, altogether this suggests that alterations in insulin/IGF-I input may be common to many, if not all neurodegenerative diseases.

It is well known that changes in growth factor and cytokine levels, including the IGFs and the IGFBPs (Torres-Aleman and Fernandez, 1998) take place at the lesioned site in all types of neurodegenerative processes, both in humans and in animal models (Isackson, 1995; Toulmond *et al.*, 1996; Feldman *et al.*, 1997; Raivich *et al.*, 1999). These changes, in most instances resulting in increased local levels of the growth factors, are considered to reflect local adaptive responses to cell death. However, it is intriguing that serum levels of insulin growth factors are changed not only in peripheral neurodegenerative diseases but even in degeneration affecting central neurons. In this regard, it has been reported that exogenously administered insulin and IGF-I can access the brain (Pardridge, 1993; Poduslo *et al.*, 1994; Reinhardt and Bondy, 1994; Carro *et al.*, 2000). While the physiological significance of these observations is currently under intense scrutiny (Aberg *et al.*, 2000; Carro *et al.*, 2000), recent findings suggest that passage of serum insulin and IGF-I through the blood–nerve barriers may be of pathological importance (Armstrong *et al.*, 2000). Furthermore, while the role of endogenous IGFs in the brain is still not clear, administration of IGFs results in potent neuroprotective effects in a great variety of experimental conditions (Feldman *et al.*, 1997; Fernandez *et al.*, 1998; Pulford *et al.*, 1999). Thus, it is conceivable that IGF therapy of neurodegenerative diseases could be implemented through peripheral administration.

It is intriguing that the pattern of changes in IGF-I, and IGFBPs in AT and CMT patients is identical (although insulin is increased only in AT patients) because these diseases are very different both in phenotype and in genotype. Similarly, animal models as diverse as diabetic rats, 3AP-ataxic rats or pcd ataxic mice (Zhang *et al.*, 1997, 1999) show identical changes in insulin, IGF-I, and IGFBPs (although insulin levels in pcd mice have not been reported). Thus, and independently of the etiology of the disease, we can distinguish two general situations: (1) Deficiency states: diseases with low insulin and/or IGF-I levels; and (2) Resistance states: diseases with high insulin and/or IGF-I levels. For the sake of clarity we do not take into account changes in IGFBPs levels because they are usually considered to be secondary to changes in insulin/IGF-I levels (Ferry *et al.*, 1999).

Deficiency states such as those found in cerebellar ataxia, amyotrophic lateral sclerosis, or stroke (Torres-Aleman *et al.*, 1996, 1998; Schwab *et al.*, 1997) may arise from a variety of causes that will ultimately lead to impaired hormone production due to either death of the hormone-synthesizing cells, cell stress, or endo-

crine disregulation. While death of pancreatic beta cells producing insulin or of hepatocytes producing IGFs is unlikely, except in neurodegeneration associated to severe endocrine diseases, our results suggests that endocrine alterations, resulting in low hormone levels may be a problem far more commonly associated to neurodegeneration than previously thought.

High IGF-I and insulin levels found in AT, CMT, and Alzheimer's disease (Tham *et al.*, 1993; Craft *et al.*, 1998) reflect a resistant state and are likely due to a loss of sensitivity of target cells to the actions of the growth factor. Although resistance may occur with low or even normal growth factor levels, high circulating levels of either insulin or IGF-I always reflect a resistant state (Jain *et al.*, 1998). Possible mechanisms leading to insulin/IGF-I resistance in neurodegenerative diseases may be varied. Genetic mutations associated to neurodegeneration may include proteins putatively involved in the insulin/IGF signalling pathway. This might be the case of the Atm protein mutated in AT patients which is a PI 3-kinase family member. PI 3-kinases are critically involved in the biological actions of IGFs and insulin (LeRoith *et al.*, 1995). Another potential interaction with the IGF pathway is the dentatorubral-pallidoluysian atrophy protein, which interacts with the insulin receptor substrate (IRS, Okamura *et al.*, 1999), which is a pivotal molecular intermediary of insulin/IGF actions (LeRoith *et al.*, 1995). Other potential causes of resistance to insulin/IGF-I may be related to pathological changes in affected cells or in their vicinity, including altered cytokine production or glucose metabolism (Jain *et al.*, 1998; Venters *et al.*, 1999). The latter processes may be involved in cell death in Alzheimer's disease since both serum insulin and IGF-I levels are elevated (Tham *et al.*, 1993; Craft *et al.*, 1998). Furthermore, it has been suggested that high serum insulin levels in Alzheimer's disease reflects an altered insulin metabolism of affected neurons (Craft *et al.*, 1998; Wickelgren, 1998). Hence, a "neuronal diabetes-like" process could be primarily involved in the development of Alzheimer's disease (Wickelgren, 1998). We can speculate that similar "resistant states" of affected nerve cells underlie high insulin/IGF levels found in AT or CMT patients. If our hypothesis is correct, patients with dentatorubral-pallidoluysian atrophy may have high serum insulin/IGF-I levels.

Although we cannot yet determine the precise mechanisms leading to either increased or decreased serum levels of IGFs in neurodegenerative diseases, at least three types of general processes can be envisaged to participate. A first one may be signals associated to the cell death process, such as cytokines (Fan *et al.*, 1998) and other cellular mediators involved in neurodegeneration. This is supported by our observation that cell death is temporally correlated with serum IGF-I levels in models of acute and of progressive neurodegeneration (see also Zhang *et al.*, 1999). The gradual normalization of serum IGF-I levels after 3AP insult is not due to transient toxic effects of the drug since lesioning of inferior olive neurons by a different method elicits a similar decrease in IGF-I levels (Fernandez *et al.*, 1998). A second possible mechanism may be metabolic derangements associated to the neurodegenerative process. For example, a high prevalence of diabetes in Friedreich's ataxia, multiple sclerosis, or Wolfram syndrome has been reported (Wertman *et al.*, 1992; Barrett *et al.*, 1995; Ristow *et al.*, 1998). As already mentioned, central derangements in insulin action could be involved in Alzheimer's disease (Craft *et al.*, 1998; Wickelgren, 1998) and may also be involved in ataxia-telangiectasia (Knittweis, 1998). In this regard, it is important to note that many endocrine diseases are associated to nerve cell death. A third possibility is that changes in insulin/IGFs levels are secondary to general illness-related changes such as alterations in food comsuption, in the sleep–wake cycle, or in physical activity. Low protein intake, sleep disorders, critical illness, and intense physical activity have all been related to changes in the insulin/IGF axis (Jones and Clemmons, 1995; Frost and Lang, 1998; Simon 1998). The relative contribution of each of these processes would be specific for each disease, resulting in a distinct pattern of changes of insulin/IGF-I in each disease.

In summary, our results indicate that changes in serum insulin, IGF-I, and/or IGF-binding proteins are associated to many different types of neurodegenerative processes. These changes are probably due to a variety of endocrine alterations associated to the disease state. However, it is tempting to speculate that in a subset of neurodegenerative diseases these changes may directly reflect pathogenic mechanisms. At any rate, our results strongly support the need to evaluate serum IGFs and possibly other circulating growth factors in all types of neurodegenerative conditions. If circulating trophic factors are found to be altered in other diseases we may start considering that endocrine/metabolic alterations are commonly associated to neurodegenerative diseases. The recent success of subcutaneous IGF-I treatment in experimental models of neurodegeneration support the notion that interventions aimed to correct these endocrine alterations may lead to new therapeutic approaches.

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Protective effects of cardiotrophin-1 adenoviral gene transfer on neuromuscular degeneration in transgenic ALS mice

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Amyotrophic lateral sclerosis (ALS) is mainly a sporadic neurodegenerative disorder characterized by loss of cortical and spinal motoneurons. Some familial ALS cases (FALS) have been linked to dominant mutations in the gene encoding Cu/Zn superoxide dismutase (*SOD1*). Transgenic mice overexpressing a mutated form of human SOD1 with a $Gly^{93}Ala$ substitution develop progressive muscle wasting and paralysis as a result of spinal motoneuron loss and die at 5 to 6 months. We investigated the effects of neurotrophic factor gene delivery in this FALS model. Intramuscular injection of an adenoviral vector encoding cardiotrophin-1 (CT-1) in $SOD1^{G93A}$ newborn mice resulted in systemic delivery of CT-1, supplying motoneurons with a continuous source of trophic factor. CT-1 delayed the onset of motor impairment as assessed in the rotarod test. Axonal degeneration was slowed and skeletal muscle atrophy was largely reduced by CT-1 treatment. By monitoring the amplitude of the evoked motor response, we showed that the time-course of motor impairment was significantly decreased by CT-1 treatment. Thus, adenovirus-mediated gene transfer of neurotrophic factors might delay neurogenic muscular atrophy and progressive neuromuscular deficiency in ALS patients.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a common neurodegenerative disease in humans, involving motoneuron loss in the cortex, brainstem and spinal cord. It typically affects adults in their fifth decade, leading to progressive muscle wasting, paralysis and death within 3 to 5 years. ALS is mainly sporadic; however, 10% of cases are familial (FALS) and have similar clinical and histopathological features. About 20% of FALS cases are associated with dominantly inherited mutations in the Cu/Zn superoxide dismutase gene (*SOD1*) (1). SOD1 cytosolic enzyme catalyzes the dismutation of superoxide radical into hydrogen peroxide and molecular oxygen, suggesting that reduced SOD activity could increase oxidative stress. However, transgenic mice expressing FALS-linked mutations in the *SOD1* gene develop severe denervating and paralytic processes that resemble ALS without loss of SOD1 activity (2–5). These observations suggest that mutant SOD1 proteins acquire a novel gain of function that might contribute to the pathogenesis. Several hypotheses have been proposed to explain this gain of function. These include increased peroxidase activity, nitration of tyrosines via formation of peroxynitrite, metal toxicity, SOD1-aggregation-mediated toxicity or inhibition of glial glutamate uptake (for reviews see 6,7). However, to date, molecular mechanisms leading to selective motoneuron degeneration remain poorly understood.

Mice expressing the $Gly^{93}Ala$ (G93A) mutation in exon 4 of the human *SOD1* gene exhibit an autosomal dominant adult onset of motoneuron disease (2). The prominent and selective loss of spinal motoneurons leads to progressive paralysis, muscle wasting, atrophy, and death at 5–6 months. These mice provide a very useful animal model of FALS and have been used for identifying therapeutic agents. Antioxidants slow down the progression of the disease but have no effect on survival (8,9), whereas inhibitors of the glutamatergic system and copper chelators delay disease onset slightly and extend survival to a small extent (8,10). A creatine diet improves motor performance and slightly protects motoneurons from oxidative damage (11). Finally, promising results were obtained through overexpression of the Bcl-2 anti-apoptotic gene or caspase inhibitors; both approaches have been shown to delay motoneuron death (12–14), suggesting that apoptosis plays an important role in ALS pathogenesis.

In the absence of a clear understanding of the pathogenic process, neurotrophic factors have been hypothesized to be able to slow down motoneuron cell death and axonal degeneration

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and also to promote muscle reinnervation (for reviews see 15,16). Beneficial effects of neurotrophic factor overexpression have been reported in spontaneous models of motoneuron disease, *wobbler* and *pmn* mice (17–20). In *pmn* mice, we recently described the impressive neuroprotective effect of cardiotrophin-1 (CT-1) (21). This cytokine of the IL-6 family has been described as a very potent neurotrophic factor for spinal motoneurons in long-term culture and protects neonatal sciatic motoneurons from axotomy-induced cell death in rats (22). Its overexpression in *pmn* mice significantly reduces degeneration of facial motoneurons and phrenic axons and preserves the terminal innervation of skeletal muscles that is grossly disturbed in untreated *pmn* mice (21). We have already shown that intramuscular adenoviral gene transfer in neonatal mice results in the efficient and long-term expression and delivery of a secreted protein (23).

Using this gene therapy approach, we have now evaluated the neuroprotective potential of CT-1 in $SOD1^{G93A}$ mice. Neonatal transgenic mice received a single injection of adenoviral vector, and both functional and histopathological parameters were evaluated to assess the effects of CT-1 gene delivery on disease progression.

RESULTS

Effects of *CT-1* gene delivery on general behavior of $SOD1^{G93A}$ mice

Neonatal transgenic $SOD1^{G93A}$ mice were injected with a total dose of 10^8 plaque forming units (PFU) of AdCT-1 or AdLacZ into five muscles (both gastrocnemii, both triceps brachii and long dorsal muscles of the trunk). We have already shown that a similar injection protocol results in long-term gene expression (20,21). In this study, gene expression was assessed from day 130–210 after gene transfer in injected muscles and also in serum. As shown in Figure 1A, adenoviral *CT-1* transcripts were detected by RT–PCR in all AdCT-1 injected muscles until very late stages. In sera, CT-1 bioactivities were measured using a ciliary ganglion neuron survival assay. At 130 days after gene transfer, the sera of all analyzed $SOD1^{G93A}$-treated mice contained elevated CT-1 bioactivities, in contrast to sera from control $SOD1^{G93A}$ mice (Fig. 1B). We thus conclude that CT-1 gene expression persisted over the course of the study.

To assess the effects of treatment on general behavior, $SOD1^{G93A}$ mice were regularly weighed (Fig. 2A). With disease progression, untreated $SOD1^{G93A}$ mice had reduced weight gain and even lost weight at end-stage, as described previously. Compared with untreated animals, AdCT-1-treated mice had a slightly reduced weight gain on the first weeks after adenoviral injection, which probably reflects the side effects of CT-1 as observed in AdCT-1-treated *pmn* mice (21). At 120 days of age however, the weight of AdCT-1-treated $SOD1^{G93A}$ mice reached values close to normal and remained higher than the weight of untreated $SOD1^{G93A}$ mice even in end-stage disease.

CT-1 delays disease onset and modestly improves survival of $SOD1^{G93A}$ mice

The appearance of the first signs of motoneuron disease in $SOD1^{G93A}$ mice was assessed by rotarod analysis. Mice were tested on a rotarod on a weekly basis from day 60–190 of life.

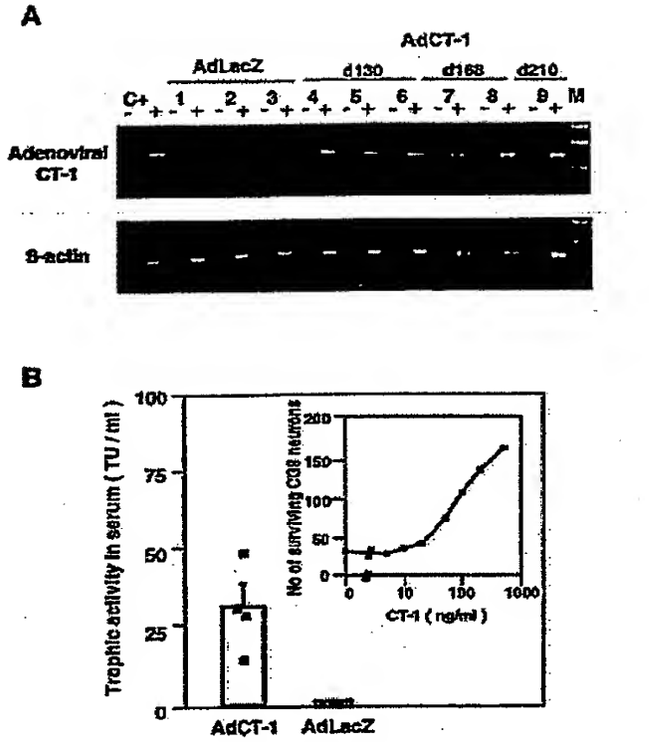

Figure 1. CT-1 expression in $SOD1^{G93A}$ mice after intramuscular gene delivery. (A) Adenoviral *CT-1* transcripts were detected by RT–PCR in gastrocnemius muscles of AdCT-1-injected mice aged 130 days (lanes 4–6), 168 days (lanes 7–8) and 210 days (lane 9). In contrast, no signal was detected in control AdLacZ-injected mice (lanes 1–3). C+, RNA from AdCT-1-infected 293 cells. Control RT–PCR reactions were performed using primers specific for β-actin. Reactions with (+) and without (–) reverse transcriptase. (B) CT-1 bioactivities in mouse sera as analyzed by a ciliary ganglion neuron survival assay. Elevated CT-1 bioactivities [in trophic units (TU) per milliliter] were detected in the sera of AdCT-1-injected $SOD1^{G93A}$ mice aged 130 days ($n = 4$) but not in control mice ($n = 4$). One trophic unit is defined as the serum dilution allowing half-maximal survival of neurons. The number of surviving neurons as a function of the concentration of recombinant CT-1 protein is shown in the inset.

The onset of motor deficit was defined as the first day a mouse could not remain for 3 min on the rotarod turning at a speed of 15 r.p.m. The average age of motor deficit onset in AdCT-1-treated $SOD1^{G93A}$ mice was 153 ± 7.8 days (mean ± SEM, $n = 7$) compared with 126 ± 8.5 days ($n = 6$) in untreated $SOD1^{G93A}$ mice (Fig. 2B). CT-1 treatment thus delayed motor deficit onset by 27 days (Fisher's test, $P = 0.039$).

As shown in Figure 2C, the CT-1 treatment prolonged the mean survival of *SOD* mice by about 13 days (mean ± SEM: untreated, 172.6 ± 4.6 days, $n = 25$; CT-1-treated, 185.9 ± 3.9 days, $n = 46$, $P = 0.034$). When compared with a small number of AdLacZ-injected $SOD1^{G93A}$ mice, the mean survival of CT-1 treated $SOD1^{G93A}$ mice also tended to improve (LacZ, 176.3 ± 5 days, $n = 12$) albeit this difference did not reach statistical significance ($P = 0.094$; Fig. 2C).

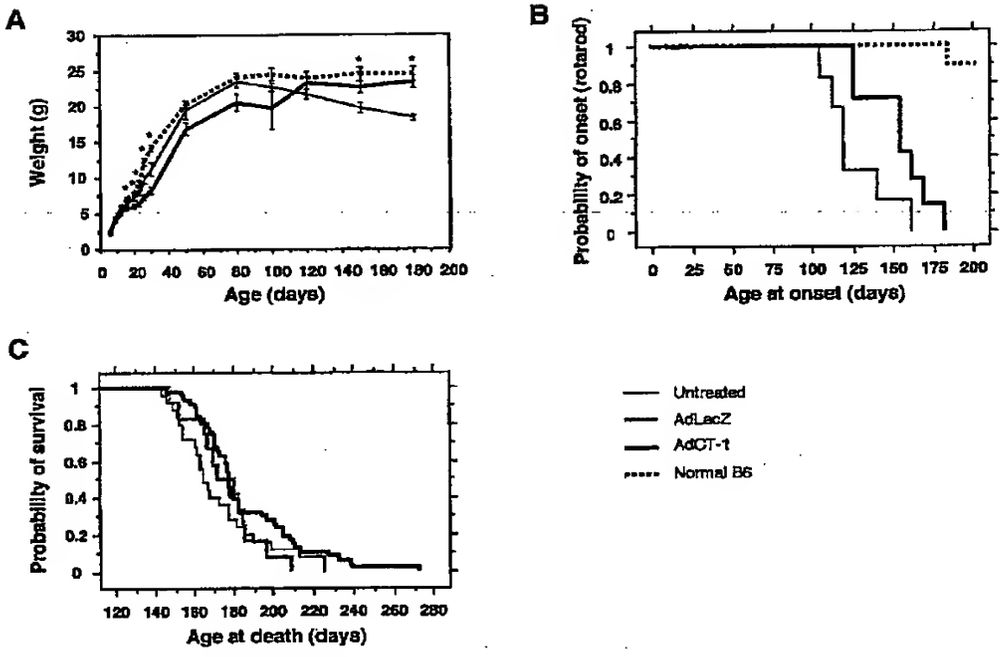

Figure 2. General behavior of *SOD1*^{G93A} mice. (A) Body weight variations in AdCT-1-treated mice and control groups. Asterisks denote the statistical difference between AdCT-1-treated mice compared with untreated animals. Average mean number of mice analyzed: wild-type, $n = 17$; AdCT-1, $n = 14$; untreated, $n = 8$. (B) Cumulative probability of onset of severe motor deficits. Motor function was assessed with the rotarod at 15 r.p.m. from day 60–190. Testing was terminated arbitrarily at 3 min. The onset of motor deficiency was defined as the day the mouse could not complete the test. CT-1 delayed onset of motor deficits by 27 days compared with untreated *SOD1*^{G93A} mice (Fisher's test, $P = 0.039$). (C) Cumulative probability of survival in *SOD1*^{G93A} mice. CT-1 improved the survival of *SOD1*^{G93A} ($n = 46$) by about 11–13 days as compared with untreated ($n = 25$) or AdLacZ-injected ($n = 12$) mice (AdCT-1 versus untreated, $P = 0.034$; AdCT-1 versus AdLacZ, $P = 0.094$).

CT-1 improves neuromuscular function of *SOD1*^{G93A} mice

We used electromyography (EMG) to characterize more precisely the progressive impairment in neuromuscular function in SOD1^{G93A}-treated mice. Changes in EMG parameters are detected far earlier than onset of tremor or motoneuron loss (24,25). In this study we recorded compound muscle action potential (CMAP) amplitudes in the gastrocnemius muscle from 80 to 180 days after injection to assess the extent of impairment of motor units after CT-1 treatment. Up to 110 days, CMAP values in gastrocnemius muscle did not differ in either CT-1-treated or control *SOD1*^{G93A} mice (Fig. 3A). Thereafter, CMAP amplitude decreased at a significantly lower rate in AdCT-1-treated compared with untreated *SOD1*^{G93A} mice (–0.44 mV/day in *SOD1*^{G93A}-untreated mice; –0.29 mV/day in CT-1-treated animals, regression analysis and Fisher's test, $P < 0.0001$). In end-stage disease (180 days), the CMAP amplitude remained significantly higher in CT-1-treated mice than in untreated *SOD1*^{G93A} mice.

Distal motor latencies were measured in parallel with CMAP in gastrocnemius muscle (Fig. 3B). Motor latencies were increased in all *SOD1*^{G93A} mice compared with normal mice. Up to 140 days of age, no significant difference was observed between untreated and AdCT-1-treated *SOD1*^{G93A} mice. At later time points however, distal motor latencies were significantly lower in CT-1-treated *SOD1*^{G93A} mice compared with untreated *SOD1*^{G93A} mice (Fisher's test, $P = 0.0003$).

Histological effects of CT-1

Histological analysis of *SOD* mice was performed at 130 days of age. The medial and lateral gastrocnemius muscles were dissected and weighed (Fig. 4). The weight of AdLacZ-treated muscles of *SOD1*^{G93A} mice was only 46% of the weight of wild-type muscles (mean ± SEM: AdLacZ, 72.2 ± 3.5 mg, $n = 5$; wild type, 156.4 ± 8.4 mg, $n = 7$). In sharp contrast, gastrocnemius muscles from AdCT-1-treated mice weighed nearly twice as much (131.7 ± 8.6 mg, $n = 10$) than those of AdlacZ-injected mice ($P < 0.001$), and only 16% less than those of wild-type mice ($P = 0.04$). These data suggest that CT-1 treatment slowed muscle atrophy.

Next, cross-sections of gastrocnemius muscles were stained for myofibrillar ATPase (Fig. 5). In untreated mice, neurogenic muscle atrophy was observed with the presence of angulated small atrophic fibers surrounded by hypertrophic fibers. Both medial and lateral parts of the gastrocnemius muscle were affected. The presence of a few muscle fibers with central nuclei indicated a certain extent of muscle regeneration. However, we did not observe changes in either the proportion of fiber type or fiber type grouping (data not shown). CT-1 treatment markedly reduced denervation atrophy. Only rare

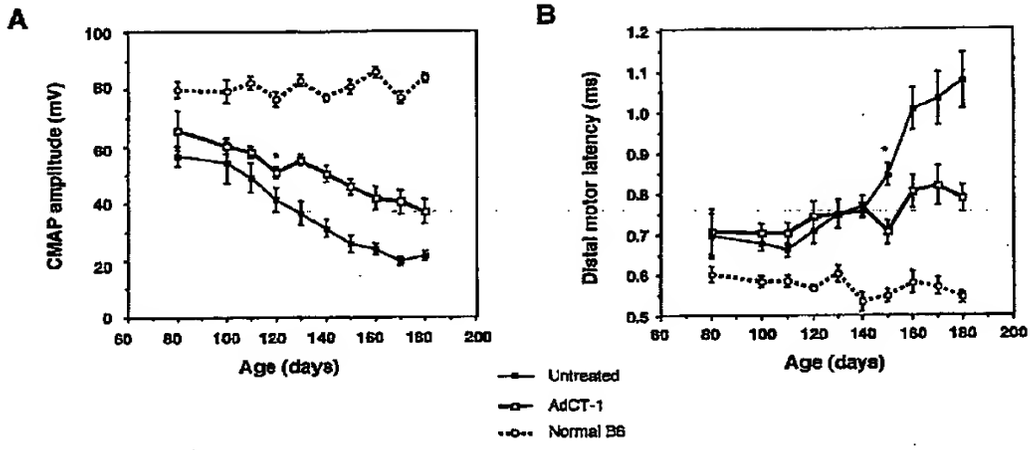

Figure 3. Time course of evoked motor response impairment in *SOD1*^{G93A} mice. (A) Evoked CMAP amplitude and (B) distal motor latency were recorded in the gastrocnemius muscle after stimulation of the sciatic nerve in AdCT-1-treated (mean number of analyzed mice, $n = 9$), untreated *SOD1*^{G93A} mice ($n = 8$) and normal littermates ($n = 7$). Measurements were made at 80 days and every 10 days from 100 to 180 days. Mean data from left and right muscles are presented (± SEM). Asterisks denote the first day on which data are significantly different between AdCT-1-treated mice and untreated *SOD1*^{G93A} mice ($P < 0.05$).

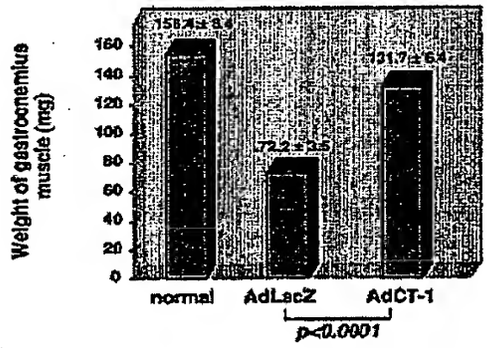

Figure 4. Weight of gastrocnemius muscles of *SOD1*^{G93A} mice aged 130 days. Note that CT-1 gene delivery significantly delayed muscle atrophy in *SOD1*^{G93A} mice as compared with control AdLacZ-injected *SOD1*^{G93A} mice ($n = 5$). AdCT-1-treated muscles ($n = 10$) were lighter than corresponding wild-type muscles ($n = 7$). Mean ± SEM.

angulated atrophic fibers were observed in the medial and lateral gastrocnemius of AdCT-1-treated mice. We next measured the fiber diameter of about 150–200 fibers per mouse in the lateral part of the gastrocnemius. A decrease in the mean fiber diameter was apparent in untreated *SOD* mice (mean ± SEM: 20.2 ± 0.8 μm, $n = 3$) as compared with normal mice (40.6 ± 0.6 μm, $n = 3$). The size distribution in untreated mice demonstrated significant atrophy with a high number of very small fibers (38.2% <12 μm) and the presence of some hypertrophic fibers with a diameter superior to normal value (4.7% >60 μm). After CT-1 treatment ($n = 3$), the fiber size distribution was much more uniform and close to normal, although the mean diameter was reduced (21.5 ± 0.3 μm).

In order to further confirm that CT-1 treatment reduced muscle denervation, muscle cross sections were treated for non-specific esterase staining and positive fibers were counted. Strong esterase activity was observed in 38.3 ± 4.8% muscle fibers from untreated *SOD1*^{G93A} mice (total number of fibers, $n = 320$), whereas only 0.6 ± 0.3% fibers ($n = 1008$) were stained in AdCT-1-treated *SOD1*^{G93A} mice and no esterase-positive fibers were observed in normal mice (Fig. 5).

Axonal degeneration in *SOD1*^{G93A} mice has been demonstrated in the ventral lumbar roots and in the phrenic nerve at end-stage disease (2,26). Here, we counted myelinated axons in phrenic nerves of mice aged 130 days (Fig. 6). Phrenic nerves of AdCT-1-treated *SOD1*^{G93A} mice contained 30% more myelinated fibers than control *SOD1*^{G93A} mice (AdCT-1, 200 ± 6, $n = 10$; AdLacZ, 153 ± 7, $n = 10$, $P < 0.001$).

DISCUSSION

Skeletal muscles transduced with an adenoviral vector were used to deliver CT-1 to motoneuron terminals, supplying them with a continuous source of biologically synthesized neurotrophic factor. We have already shown that such gene therapy approach was effective in slowing down motoneuron cell body and axonal degeneration in the *pmn* mice (21). Untreated, these mice suffer from progressive motor neuronopathy with prominent axonal degeneration and muscle atrophy. Lower motoneuron cell death is observed in end-stage disease mainly in the facial nucleus and to a lesser extent in the lumbar spinal cord (17,20,27). However, the mechanism by which *pmn* motoneurons undergo cell death is unknown since the *pmn* mutation remains undiscovered. Here, mice transgenic for a mutated form of the human *SOD1* gene (*SOD1*^{G93A}) associated with FALS provided an ideal model to test our experimental therapeutic approach for ALS. Loss of lower motoneurons in the spinal cord leads to hindlimb tremor, muscle wasting and progressive muscle atrophy resulting in death at 5 to 6 months of age. Direct intramuscular injection of the AdCT-1 vector results in efficient and stable expression of

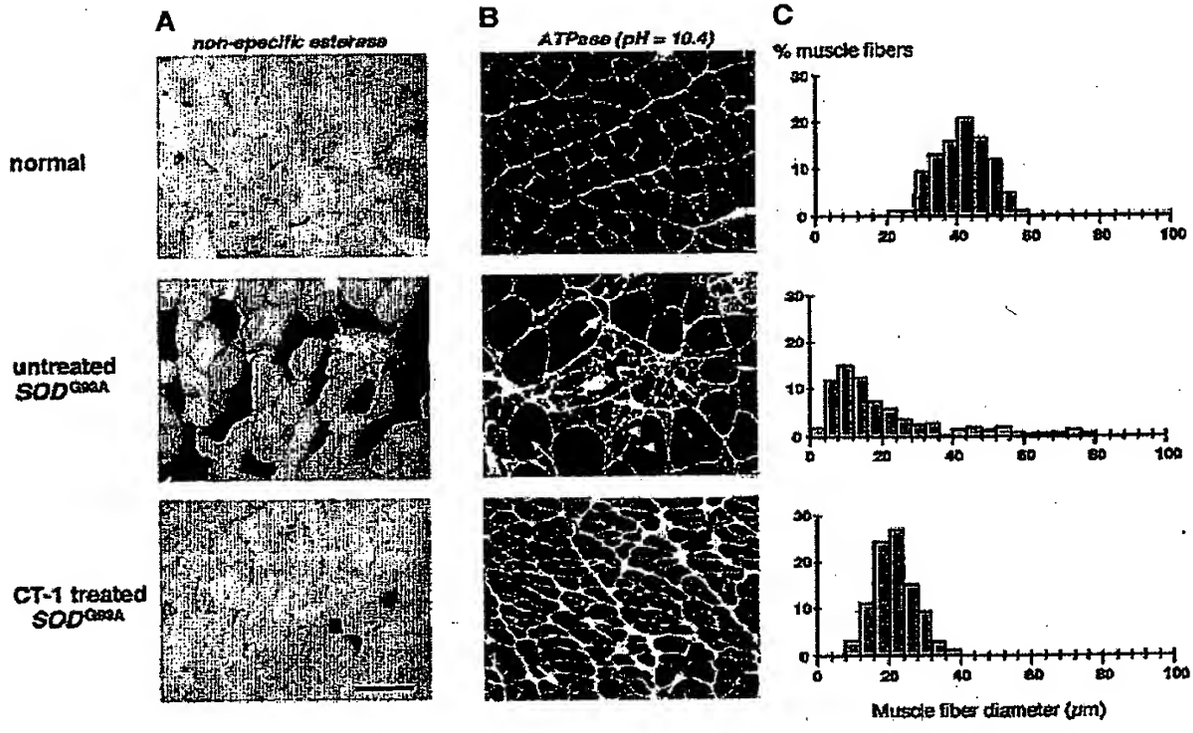

Figure 5. Histological analysis of denervation-induced muscle atrophy in untreated and CT-1-treated *SOD1*^{G93A} mice. Cross-sections of gastrocnemius muscles of mice aged 130 days were stained for non-specific esterase (A) and alkaline ATPase (B) histochemistry. (A) Numerous denervated fibers as indicated by strong esterase activity were present in untreated *SOD1* ^{G93A} mice ($n = 3$). In contrast, stained fibers were rare after CT-1 treatment ($n = 3$). (B) In untreated *SOD1*^{G93} mice, large groups of angulated and atrophic muscle fibers (thick arrows) were observed after ATPase staining. Most often, hypertrophic fibers (thin arrow) were present in atrophic regions. Signs of regeneration were also evident with fibers showing central nuclei (arrowheads). CT-1 treatment markedly attenuated denervation atrophy. No gross muscle fiber abnormalities were observed in AdCT-1-treated *SOD* mice, although fiber area was smaller than in normal mice ($n = 3$). (C) Morphometric analysis confirmed changes in the distribution of muscle fiber diameter. Megahistograms were constructed by combining three histograms from the lateral gastrocnemius of each group. Scale bar, 100 μm.

CT-1 in muscles for at least 210 days, resulting in CT-1 activity in bloodstream. General behavior of AdCT-1-treated mice, as measured by body weight, was improved in end-stage disease. Furthermore, CT-1 delayed the onset of severe motor deficits as evaluated by improved performance in the rotarod test. The decline of EMG parameters was also significantly delayed. Both CMAP amplitude and distal motor latency were improved by AdCT-1 injection. We conclude that CT-1 overexpression is able to slow down degradation of the neuromuscular function in FALS transgenic mice.

At the age of 130 days, the weight of gastrocnemius muscle in untreated *SOD1*^{G93A} mice was half that in normal mice in accordance with previous observations (28). In contrast, muscle atrophy was slowed dramatically after CT-1 treatment, although some cytokine cachexic effects have probably resulted in loss of muscle fiber area (29,30). Histological observations confirmed that neurogenic atrophy was clearly reduced by CT-1 treatment. Several hypotheses might explain this effect. CT-1 expression may have stimulated sprouting which might increase innervation of muscles and protect them against atrophy. However, we did not observe any changes in the fiber typing pattern in gastrocnemius muscles. Furthermore, our observation of terminal innervation in intercostal and abdominal muscles, either at the 130th day or at the terminal stage of the disease (data not shown), did not support this hypothesis since sprouting or branching of terminal axons did not seem to be increased compared to untreated mice. Another hypothesis is that CT-1 may have protected muscles from denervation by slowing down motoneuron and axonal degeneration. A denervation marker, strong esterase activity (31), was evident in untreated *SOD* mice, whereas it was nearly absent in AdCT-1-treated mice. We also observed an increased number of myelinated axons in phrenic nerves of CT-1-treated mice compared with control AdLacZ-injected *SOD1*^{G93A} mice, providing evidence that axonal degeneration was clearly reduced by CT-1 treatment. These results are consistent with our previous observations in *pmn* mice, where the terminal innervation pattern appeared to be preserved by CT-1 gene delivery though no marked reinnervation was noted (21). Furthermore, raised CMAP amplitudes in AdCT-1-treated mice indicate

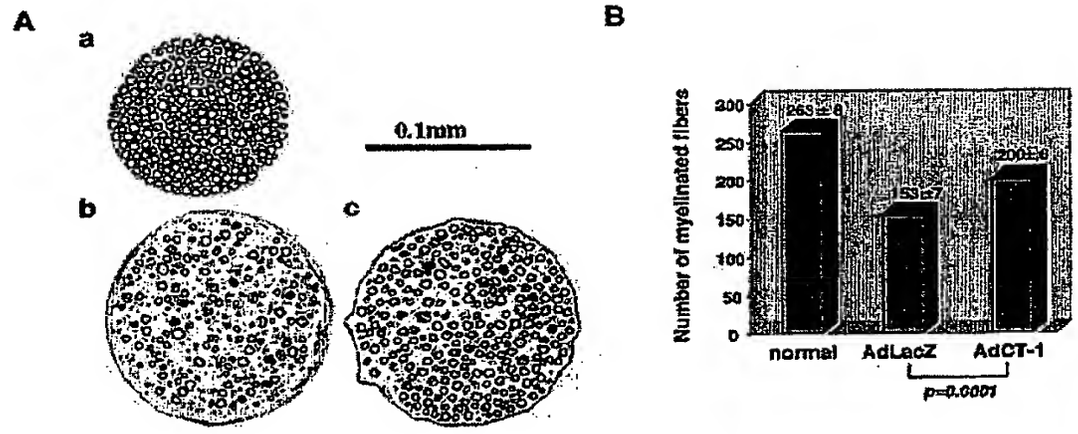

Figure 6. Phrenic nerve axonal counts in 130-day-old $SOD1^{G93A}$ mice. (A) Histological analysis of phrenic nerves from a normal C57Bl/6 mice (a), AdLacZ-injected $SOD1^{G93A}$ mice (b) and AdCT-1-treated mice aged 130 days (c). (B) Intramuscular CT-1 gene delivery to $SOD1^{G93A}$ mice ($n = 10$) resulted in a significantly greater number of surviving myelinated axons compared with control AdLacZ-injected $SOD1^{G93A}$ mice ($n = 10$). Compared with wild-type littermates ($n = 4$), CT-1 treatment reduced axonal degeneration in FALS mice by 40%. Mean ± SEM.

that more muscle fibers were innervated (24). Altogether, these results suggest that CT-1 delays degeneration distally at the nerve terminals and neuromuscular junctions, and thereby prevents the gradual decline in the size of the motor units. Finally, myotrophic effects of CT-1 may have contributed to reduce muscle atrophy. Such myotrophic effects of CT-1 have been well described in cardiac muscle (32–34), but have not yet been reported in skeletal muscle. However, they have been described for the related cytokines CNTF and LIF after nerve section (30,35) and, for CNTF, also in *wobbler* mice (36). It is also known that the LIFRβ and gp130 subunit receptors of the CT-1 receptor are expressed by skeletal muscle and that their expression is increased in denervated muscle (35).

Recently, a myoblast-based GDNF gene therapy was applied in $SOD1^{G93A}$ mice (37). Myoblasts infected with GDNF retroviral vectors, and thus secreting this factor, were grafted into hindlimb muscles of $SOD1^{G93A}$ mice. This prevented loss of large diameter spinal motoneurons and delayed the onset of the disease. However, no effect on survival was reported. In our study, the mean lifespan of $SOD1^{G93A}$ was increased after AdCT-1 injection although the observed gain of two weeks was relatively small. This result contrasts with the dramatic protective effects of CT-1 on axonal degeneration and muscle atrophy. Several hypotheses might explain this discrepancy. First, survival pathways initiated by CT-1 might not be efficient enough to circumvent the apoptotic events activated very early in the $SOD1^{G93A}$ disease (14). Second, the increased circulating CT-1 levels we measured in late stage animals might not have been available at sufficient amounts to degenerating motoneurons. Indeed, axonal transport, which is impaired in various ALS transgenic mice (38,39), might have lead to insufficient supply of the trophic factor to cell bodies thereby limiting its action. Finally, there is increasing evidence that subpopulations of motoneurons differ in their vulnerability to degeneration (40) and also in their response to trophic factors (41). In cell culture or during normal development for instance, CT-1 seems to be required only for the survival of subpopulations of motoneurons (42). In $SOD1^{G93A}$ mice, some fast-type neuromuscular synapses are already lost around day 50, whereas slow-type synapses resist until late phases of disease (40). Our results indicate that CT-1 treatment did not significantly improve the earliest electromyographic abnormalities of $SOD1^{G93A}$ mice but had pronounced effects on disease progression. It can thus be speculated that CT-1 protected selected subpopulations of degenerating motoneurons or neuromuscular synapses.

In conclusion, we demonstrate that CT-1 gene delivery has neuroprotective effects in a transgenic mouse model of FALS. The results previously observed in the *pmn* model were confirmed in these mice. The marked slowing of axonal degeneration and muscle denervation leads to a protection against the loss of neuromuscular function and a delayed onset of severe motor dysfunction. These results suggest that a similar therapy resulting in continuous neurotrophic factor production by transduced skeletal muscles could also improve the course of ALS in humans. Associated perhaps with other drugs that protect motoneuron cell bodies from apoptosis, *CT-1* gene therapy could be one of the promising innovative treatments that are anxiously awaited in ALS, today a largely untreatable disease with an appalling prognosis.

MATERIALS AND METHODS

Animals

Male transgenic mice with the G93A human *SOD1* mutation (G1 line) were provided by Transgenic Alliance (Saint-Germain-sur-L'Arbresle, France). The gene copy number was confirmed by Southern blot to be about 13–19 as previously shown (2). Male transgenic mice were mated with background-matched B6SJL/F_1 wild-type females (Iffa Credo, L'Arbresle, France). The progeny were genotyped by the polymerase chain reaction (PCR) amplification of toe DNA from 3-day-old animals. PCR primers used were: exon 3, 5'-TTCTGTTCCCTTCTCACTGT-3'

and 5′-TCCCCTTTGGCACTTGTATT-3′; exon 5, 5′-TGTTGGGAGGAGGTAGTGATTA-3′ and 5′-AGCAGAGTTGTGTTAGTTTTAG-3′. Expected product sizes for exons 3 and 5 were 500 and 760 bp, respectively. Internal PCR control was achieved by amplifying part of the mouse globin gene with primers, 5′-GATCATGACCGCCGTAGG-3′ and 5′-CATGAACTTGTCCCAGGCTT-3′ in the same reaction. Mice were killed at various time points for *CT-1* gene expression analysis and histological measurements. All animal experiments were carried out in accordance with institutional guidelines for care and use of laboratory animals.

Intramuscular injection of adenoviral vectors

Construction of AdCT-1 and AdLacZ has been described previously (21,43). Briefly, the AdCT-1 vector drives the expression of murine CT-1 cDNA fitted with the βNGF signal peptide under the control of the RSV LTR promoter. Viruses were amplified in 293 cells and purified on CsCl gradients according to standard methods (44). Titers were 3×10^{11} PFU/ml for AdCT-1 and 1.8×10^{11} PFU/ml for AdLacZ. Neonate mice (5–6 days) were briefly anesthetized by hypothermia. Vectors were diluted to 1×10^{8} PFU/100 µl of PBS and injected into three muscle groups, the gastrocnemius (25 µl each), the triceps brachii (15 µl each) and the long muscles of the dorsal trunk (20 µl).

CT-1 gene expression

Transgene expression was determined over the course of the disease. The gastrocnemius muscles were dissected and homogenized in RNA-B reagent (Bioprobe, Montreuil, France) for total RNA purification. RT–PCR analysis of adenoviral *CT-1* transcripts was performed with the specific primers described previously (21). Control RT–PCR reactions were carried out using β-actin primers. CT-1 bioactivity in sera was determined at 130 days of age using a survival assay of ciliary ganglion neurons essentially as detailed previously (45). Briefly, ciliary ganglion neurons from 8-day-old chicken embryos were isolated, seeded in 96-well culture dishes and cultured in chemically defined medium to which sera or recombinant CT-1 protein (R&D, Abingdon, UK) were added at various dilutions. Neuronal survival in triplicate wells was assessed 48 h after plating by adding the vital dye MTT and counting the blue cells having metabolized the MTT.

Onset of motor dysfunction

A rotarod (Letica LE8200, Italy) was used to evaluate motor function. Mice were placed on the rotating rod at 15 r.p.m. The time each mouse remained on the rod was recorded. If the mouse remained on the rod for 3 min, the test was stopped and scored as 3 min. The test was performed twice and only the maximum time was taken into account. Assays were done on a weekly basis from day 60–190. The onset of motor deficits was defined as the first day a mouse could not remain on the rotarod for 3 min.

Electrophysiological recordings

Evoked CMAP amplitudes were evaluated with a RACIA (M.E.I., Montreuil, France) EMG apparatus as described previously (24). Mice were deeply anesthetized with 60 µg/g sodium pentobarbital. The sciatic nerve was stimulated by single 0.2 ms supramaximal pulses through a concentric needle electrode (Dantec, 9013R0312, diameter 0.3 mm), and CMAPs were recorded from the medial part of the gastrocnemius with the same type of electrode. The peak-to-peak amplitudes and distal motor latencies of the evoked responses were measured three times in left and right muscles and averaged. Measurements were repeated every 10 days from 80 to 180 days of age in normal and $SOD1^{G93A}$ mice.

Histological examination

To evaluate nerve and muscle degeneration, mice were killed at 130 days of age and during end-stage disease. Nerves were prepared as described previously (20). Briefly, mice were deeply anesthetized and phrenic nerves fixed *in situ* with 2.5% glutaraldehyde, 20 mg/ml sodium cacodylate before dissection. Nerves were post-fixed with osmic tetroxide and embedded in epoxy resin. 3 µm thick cross-sections, taken at a similar level from the diaphragm, were stained with p-phenylenediamine. Myelinated fibers were counted under light microscopy.

Gastrocnemius muscles were dissected, weighed and frozen immediately in cooled isopentane and stored at –80°C. Serial 10 µm thick sections were stained for myofibrillar ATPase under acid (pH 4.35; pH 5.53) and basic (pH 10.4) conditions using standard methods to allow identification of muscle fiber types I and II (46). ATPase stained sections were imaged using an image analysis system (Samba 2005 TITN, Alcatel, France). The size of muscle fibers was assessed by measuring the 'smallest fiber diameter'. Typically, 150–200 fibers were analyzed for each muscle. Some cross-sections were also stained for non-specific esterases using the α napthyl acetate method. Briefly, sections were fixed in citrate-acetone-formaldehyde solution and incubated in cholinesterase inhibitor (eserine 10 µM) for 30 min at 4°C. After washing, sections were incubated in staining medium (0.1 M Trizma maleate, 2 mM sodium nitrite, 0.3 mg/ml Fast Blue BB base, 0.25 mg/ml α-napthyl acetate) for 30 min at 37°C protected from light. After intensive washes, slides were counterstained in hematoxylin solution and mounted in aqueous medium.

Statistical analysis

Statistical analysis was performed using repeated measures analysis of variance (ANOVA). Differences between individual groups were evaluated using the Fisher post-hoc test.

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Charcot–Marie–Tooth disease neurofilament mutations disrupt neurofilament assembly and axonal transport

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Charcot–Marie–Tooth disease (CMT) is the most common inherited disorder of the peripheral nervous system, and mutations in neurofilaments have been linked to some forms of CMT. Neurofilaments are the major intermediate filaments of neurones, but the mechanisms by which the CMT mutations induce disease are not known. Here, we demonstrate that CMT mutant neurofilaments disrupt both neurofilament assembly and axonal transport of neurofilaments in cultured mammalian cells and neurones. We also show that CMT mutant neurofilaments perturb the localization of mitochondria in neurones. Accumulations of neurofilaments are a pathological feature of several neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease, dementia with Lewy bodies, and diabetic neuropathy. Our results demonstrate that aberrant neurofilament assembly and transport can induce neurological disease, and further implicate defective neurofilament metabolism in the pathogenesis of human neurodegenerative diseases.

INTRODUCTION

Charcot–Marie–Tooth disease (CMT) is a group of neuropathies that constitute the most common inherited disorders of the peripheral nervous system (1). Mutations in a number of genes cause CMT (2–6), and mutations in neurofilament light chain have recently been demonstrated to cause a form of type- 2 CMT (7,8).

Neurofilaments are the major intermediate filaments of neurones and in most mature neurones contain three subunit proteins: neurofilament light, middle and heavy chains (NF-L, NF-M and NF-H). As with other members of the intermediate filament family, neurofilament proteins share a common structural organization that comprises a central α-helical rod domain that is flanked by N-terminal head and C-terminal tail domains (9,10). The central rod domains facilitate the formation of coiled-coil oligomers that can then assemble into filaments of 10 nm diameter; the N-terminal head domains are believed to regulate the assembly properties of the filament, and the C-terminal tail domains of NF-M and NF-H (which are longer than that of NF-L) form side-arms that project from the filament and appear to form interconnections between neurofilaments and other axoplasmic organelles (11–14).

The mutations in NF-L that are associated with CMT involve a two-base conversion at codon 8 that results in a proline-to-arginine substitution and a single conversion at codon 333 that substitutes proline for glutamine (7,8). Codon 8 resides within the head domain, whereas codon 333 is situated in coil 2B of the central rod domain of NF-L. Both Pro8 and Gln333 are highly conserved in mammals and *Xenopus*, which suggests that they are structurally and/or functionally important. However, the effects that these different CMT mutations have on neurofilament assembly and architecture are not known. Here, we demonstrate that both CMT NF-L mutant proteins disrupt neurofilament assembly and axonal transport.

RESULTS

We initially studied the effects of the NF-L Pro8Arg and Gln333Pro mutations (NF-L^{Pro8Arg} and NF-L^{Gln333Pro}) on neurofilament assembly in transfected SW13− cells. These cells do not express endogenous intermediate filament proteins

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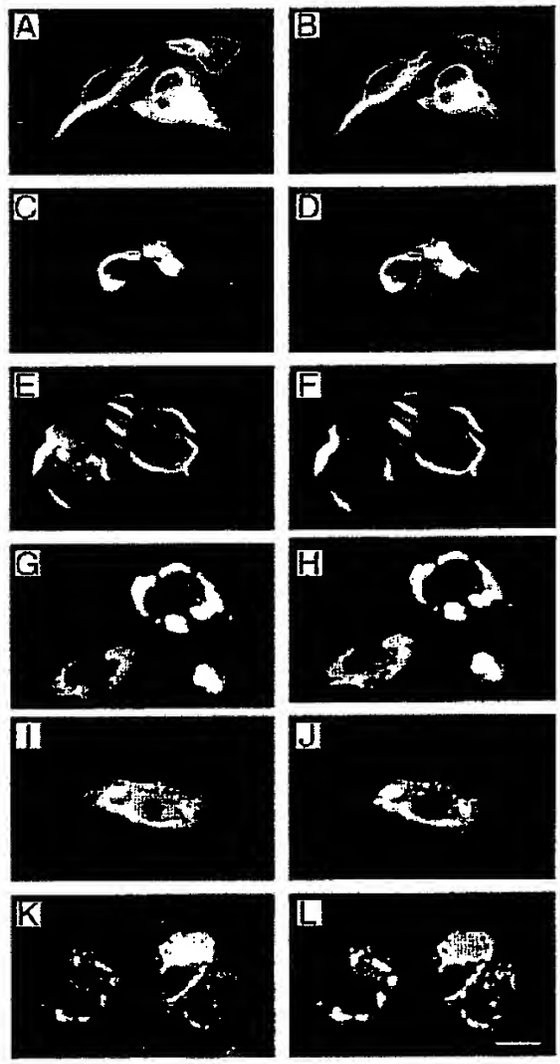


Figure 1. Assembly properties of human and rat NF-L^{Pro8Arg} and NF-L^{Gln333Pro} in SW13− cells. Cells were co-transfected with rat NF-M and NF-H, and human NF-L (A and B) human NF-L^{Pro8Arg} (C–F) human NF-L^{Gln333Pro} (G and H) rat NF-L^{Pro8Arg} (I and J) or rat NF-L^{Gln333Pro} (K and L). (A), (C), (E), (G), (I) and (K) show NF-L labelled with NA1214; (B), (D), (F), (H), (J) and (L) are labelled for NF-M/NF-H using antibody SMI32. Scale bar = 25 μm.

and so have been widely used to investigate neurofilament assembly properties (15–18). Transfection of human wild-type NF-L with NF-M and NF-H induced the formation of typical intermediate filament networks (Fig. 1A and B). However, transfection of either NF-L^{Pro8Arg} or NF-L^{Gln333Pro} with NF-M and NF-H disrupted these networks and led to the formation of abnormal structures containing all three proteins (Fig. 1C–H). These varied in appearance, with both smaller, punctate bodies and larger aggregates being discernible; flame-shaped structures were often seen with NF-L^{Pro8Arg} (Fig. 1E and F).

There is evidence that human NF-L displays different assembly properties to rodent NF-L in SW13− cells (19). Since Pro8 and Gln333 are highly conserved, we created rat CMT NF-L^{Pro8Arg} and NF-L^{Gln333Pro} mutants and studied their assembly properties in SW13− cells. However, these rat CMT mutants also disrupted neurofilament assembly in a similar manner to the human mutants (Fig. 1I–L).

We next investigated the effects of the CMT mutations on neurofilament assembly *in vivo* by transfection of the mutants into rat cortical neurones. To distinguish transfected from endogenous NF-L, we utilized the human NF-L clones and detected these with a human-specific NF-L antibody. Transfected human wild-type NF-L localized to cell bodies, axons and dendrites, and higher-magnification images revealed that it co-assembled with endogenous NF-M into filaments (Fig. 2A–C). These findings are in agreement with previous studies of neurofilaments transfected into rat neurones (17,20–22). However, transfection of either NF-L^{Pro8Arg} or NF-L^{Gln333Pro} both disrupted neurofilament architecture. In cells transfected with the CMT mutants, transfected NF-L co-localized with endogenous NF-M (although occasional images indicated the presence of some NF-L-only-containing structures), but these mutant neurofilament proteins accumulated in the cell body or the cell body and proximal regions of neurites; the CMT mutants were rarely seen in more distal regions of axons. Abnormal neurofilament aggregates, reminiscent of those seen in the SW13− cells, were also commonly seen in cell bodies, and these were particularly noticeable in cells expressing higher levels of transfected CMT NF-L (as judged by intensity of fluorescence) (Figure 2D–G). Such aggregates were never seen in cells expressing wild-type NF-L.

Cells that are affected in CMT include sensory neurones in the dorsal root ganglion (DRG). We therefore studied the effect of expressing wild-type and CMT mutant NF-Ls in cultured DRG neurones. Transfected wild-type NF-L localized to cell bodies and axons in a similar fashion to that seen in cortical neurones (Figure 3A and B). However, both NF-L^{Pro8Arg} and NF-L^{Gln333Pro} again accumulated in cell bodies and proximal axons. In the DRG neurones, the cell body accumulations generally appeared as a single aggregate (Figure 3C–F).

To gain some insight into the polymeric form of NF-L^{Pro8Arg} and NF-L^{Gln333Pro}, we prepared Triton X-100-insoluble and -soluble fractions from SW13− cells co-transfected with NF-M and NF-H and wild-type NF-L, NF-L^{Pro8Arg} or NF-L^{Gln333Pro}, and analysed the distribution of the individual neurofilament proteins. Neurofilament proteins that are assembled into intermediate filaments are present in the Triton X-100-insoluble fraction of such preparations. In these experiments, NF-L^{Pro8Arg} and NF-L^{Gln333Pro} fractionated to the Triton X-100-insoluble component at both 15 000*g* (average) and 100 000*g* (average) (Fig. 4). The pelleting of the mutant NF-Ls suggests that they are assembled into a polymeric form of higher order than the tetrameric stage. Thus, both CMT mutant proteins disrupt normal neurofilament assembly in SW13− cells and neurones, but their co-localization with NF-M/NF-H and presence in a Triton X-100-insoluble fraction indicate that this does not preclude co-assembly with other neurofilament proteins.

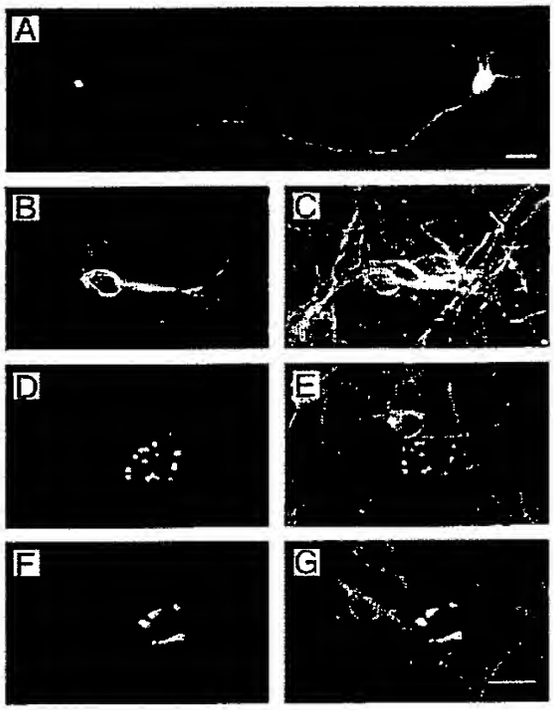


Figure 2. Assembly properties of NF-L^{Pro8Arg} and NF-L^{Gln333Pro} in rat cortical neurones. Cells were transfected with human clones for wild-type NF-L (A–C) NF-L^{Pro8Arg} (D and E) or NF-L^{Gln333Pro} (F and G). (A), (B), (D) and (F) are labelled for NF-L using human-specific monoclonal antibody anti-NF70; (C), (E) and (G) are labelled for NF-M using rabbit antibody NF-M-Cterm. Scale bars = 25 μm.

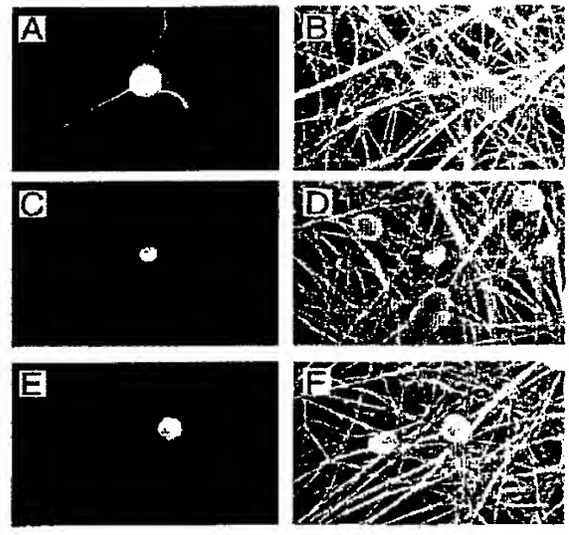


Figure 3. Assembly and transport properties of NF-L^{Pro8Arg} and NF-L^{Gln333Pro} in rat DRG neurones. Cells were transfected with human clones for wild-type NF-L (A and B) NF-L^{Pro8Arg} (C and D) or NF-L^{Gln333Pro} (E and F). (A), (C) and (E) are labelled for NF-L using human-specific monoclonal antibody anti-NF70; (B), (D) and (F) are labelled for NF-M using rabbit antibody NF-M-Cterm. Scale bar = 40 μm.

Neurofilament proteins are synthesized in cell bodies and then transported into and through axons (23–26). The restriction of NF-L^{Pro8Arg} and NF-L^{Gln333Pro} to cell bodies and proximal regions of axons in the transfected cortical and DRG neurones suggests that the mutants somehow disrupt axonal transport of neurofilaments. We therefore analysed the effects of the two CMT mutants on axonal transport of neurofilaments using a previously described assay (17). This assay involves monitoring movement of enhanced green fluorescent protein (EGFP)-tagged NF-M in transfected cortical neurones by fixation of cells at set time points and measurement of the distance travelled by the fluorescent EGFP–NF-M front. EGFP–NF-M co-transfected with wild-type human NF-L travelled at a rate of ~80 μm/h (Fig. 5A). This is in close agreement with that previously calculated by us for cells transfected with EGFP–NF-M alone (17), and demonstrates that co-transfection with NF-L has little, if any, effect on the movement of EGFP–NF-M. However, co-transfection of either NF-L^{Pro8Arg} or NF-L^{Gln333Pro} significantly reduced the distance travelled by EGFP–NF-M (Fig. 5B).

In a complementary study, we also analysed the effect of the CMT mutant NF-Ls on axonal transport of mitochondria by quantifying the distribution of mitochondria in a defined segment of axons. This approach has recently been successfully used to study the effect of transfected tau on mitochondrial transport (27). To do so, we transfected DRG neurones with a marker for mitochondria (*Discosoma* red fluorescent protein fused to the mitochondrial targeting sequence from subunit VIII of human cytochrome *c* oxidase, DsRed2–Mito) (28) either alone or with human wild-type NF-L, NF-L^{Pro8Arg} or NF-L^{Gln333Pro}. Cells transfected with DsRed2–Mito alone revealed that mitochondria were present in cell bodies and throughout the length of neurites (Fig. 6A and B) which is in agreement with other studies on the distribution of mitochondria in neurones (27). Similar images were obtained from cells co-transfected with DsRed2–Mito and wild-type NF-L (Fig. 6C and D). However, mitochondria in cells co-transfected with DsRed2–Mito and either NF-L^{Pro8Arg} or NF-L^{Gln333Pro} were clustered in cell bodies and proximal axons, with markedly fewer present in more distal regions of axons (Figure 6E–H). To quantify this, we counted the number of mitochondria present in a defined segment of axons, 50–100 μm from the cell body. These studies revealed that significantly fewer mitochondria were present in this region in cells co-transfected with either NF-L^{Pro8Arg} or NF-L^{Gln333Pro} compared with cells transfected with wild-type NF-L (Fig. 6I). Thus, the two CMT mutant neurofilament proteins inhibit axonal transport of both neurofilaments and mitochondria.

DISCUSSION

An increasing body of evidence suggests that disruptions to neurofilament metabolism are part of the disease process in a number of neurodegenerative diseases. Two NF-L mutations have so far been described as causative for CMT2. However,

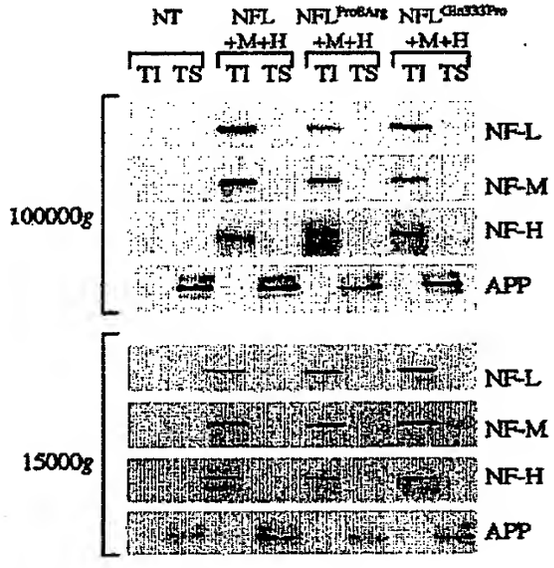


Figure 4. Biochemical properties of NF-L^{Pro8Arg} and NF-L^{Gln333Pro}. Triton X-100-insoluble (TI) and -soluble (TS) fractions were prepared from SW13– cells transfected with NF-M and NF-H, and wild-type human NF-L, NF-L^{Pro8Arg} or NF-L^{Gln333Pro}. NT are non-transfected cells. Fractions were prepared at 15 000*g* (average) and 100 000*g* (average) as indicated. Fractionation of APP to the Triton X-100-soluble component is shown as a control. Similar results were obtained using rat NF-L.

NFL mutations in several further CMT families have now been identified and probably account for ~2% of CMT kindreds (V. Timmerman, personal communication). Aside from these NF-L CMT2 mutations, mutations in NF-H have been shown to be a risk factor for amyotrophic lateral sclerosis (ALS) (29–31), and recently a mutation in NF-M has been linked to familial Parkinson's disease (32). In addition, accumulations of neurofilaments are a pathology of several neurodegenerative diseases including ALS, Alzheimer's disease, Parkinson's disease, dementia with Lewy bodies and diabetic neuropathy (for reviews see 26,33,34). Also, overexpression of neurofilament proteins, including peripherin, in transgenic mice can provide models of ALS (35–38), and modulating neurofilament expression alters disease progression in transgenic models of ALS caused by mutant superoxide dismutase 1 (SOD1) (39–42). Interestingly, transgenic mice expressing a mutant NF-L in which codon 394 (close to the CMT2 codon 333 mutation) is mutated from leucine to proline develop a particularly aggressive form of motor neurone disease (37). Finally, overexpression of peripherin has recently been shown to induce apoptotic death of neurones that is mediated by the proinflammatory cytokine tumour necrosis factor α (TNF-α) (43).

Disruption of axonal transport of neurofilaments is one of the earliest pathological features seen in these transgenic models of ALS (44–46), and this suggests that it is a primary pathogenic event that induces disease and not some end-stage epiphenomenon. It is thus notable that both CMT2 mutant NF-Ls disrupt axonal transport of neurofilaments.

We also demonstrate that these mutant NF-Ls perturb transport of mitochondria into and through axons, leading to their accumulation in cell bodies and proximal axons. Neurones are highly polarized cells with organelles, vesicles and other protein complexes requiring transportation to their appropriate final destinations following synthesis. The distances involved in this transport can be considerable; for example, human motor neurone axons can exceed 1 m in length. The accumulation of mitochondria in cell bodies and proximal axons strongly suggests that the supply of metabolic energy to more distal regions of axons and dendrites is impaired in CMT. This will perturb the functioning of kinesin and dynein family motors, which both require ATP, and, as such, disrupt the supply of essential axoplasmic components. It is likely that this disruption is mechanistic in neuronal cell death in CMT. Indeed, mutation of KIF1bβ (a molecular motor protein) has been shown to cause another form of type 2 CMT (47), and this further implicates axonal transport in the disease process.

Our results presented here, showing that two NF-L mutations linked to CMT disrupt neurofilament assembly and axonal transport, demonstrate that defective neurofilament metabolism can induce neurological disease.

MATERIALS AND METHODS

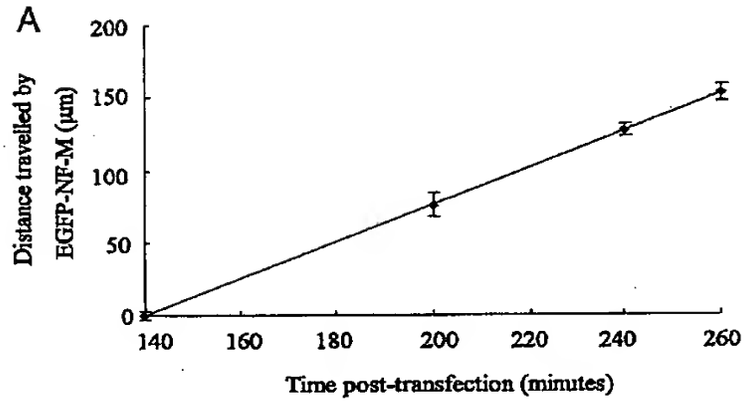
All of the experiments described in the Results section were performed at least three times with similar results.

Plasmids

Expression plasmids for human and rat neurofilaments including EGFP–NF-M were as previously described (17,48). Mutants were prepared using Stratagene Quickchange or Chameleon kits according to the manufacturers' instructions Mutagenic oligonucleotides were 5′-GTTCCTTCAGCTACG-AGAGGTACTACTCGACCTCC-3′ and 5′-GGAGGTCGAGT-AGTACCTCTCGTAGCTGAAGGAAC-3′ (human NF-L^{Pro8Arg}); 5′-GAAGCGCTGGAGAAGCCGCTGCAGGAGCTGGAGG-3′ and 5′-CCTCCAGCTCCTGCAGCGGCTTCTCCAGCGCTTC-3′ (human NF-L^{Gln333Pro}); 5′-GTTCGTTCAGCTACGAGAGGT-ACTTTTCGACCTCC-3′ (rat NF-L^{Pro8Arg}); 5′-GAAGCTCTA-GAGAAGCCGCTGCAGGAGCTGGAG-3′ (rat NF-L^{Gln333Pro}). Mitochondria were visualized by transfection with the vector pDsRed2–Mito (Clontech).

Cell culture, transfection and immunofluorescence microscopy

SW13– cells were grown in DMEM containing 10% (v/v) fetal bovine serum (FBS) supplemented with 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen) and transfected using Lipofectamine 2000 (Invitrogen), essentially according to the manufacturer's instructions. For immunofluorescence studies, cells were cultured in 12-well plates (Falcon) on glass coverslips. Primary cortical neurones were obtained from E18 rat embryos and cultured on glass coverslips coated with poly-D-lysine in 12-well plates in Neurobasal medium and B27 supplement (Invitrogen) containing 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. Cells were cultured for 7 days, and under these conditions were



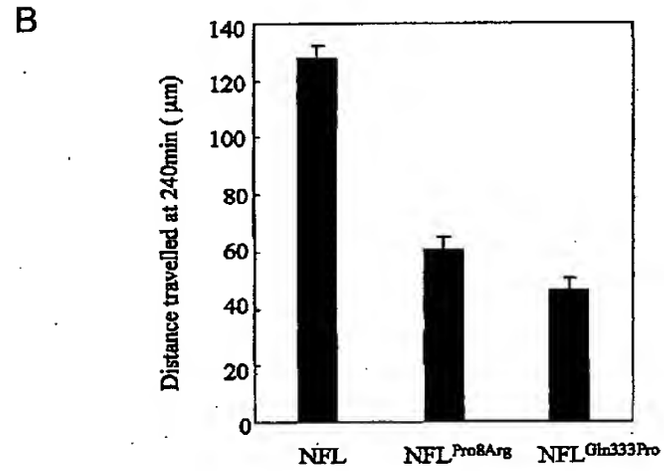


Figure 5. Neurofilament transport in rat cortical neurones. Cells were co-transfected with rat EGFP–NF-M and human wild-type NF-L, NF-L^{Pro8Arg} or NF-L^{Gln333Pro}, and the distance travelled by EGFP–NF-M was then calculated. (A) Rate of transport of EGFP–NF-M in cells co-transfected with wild-type NF-L. (B) Distance travelled by EGFP–NF-M at the 240 min time point in cells co-transfected with wild-type NF-L, NF-L^{Pro8Arg} or NF-L^{Gln333Pro}. One-way ANOVA tests revealed significant differences ($P < 0.001$) in the distances travelled by EGFP–NF-M between wild-type NF-L and NF-L^{Pro8Arg} or NF-L^{Gln333Pro} co-transfected cells. Error bars are SEM.

almost exclusively neurones, as previously described by us and others (17,49). Cortical neurones were transfected using a Promega calcium phosphate Profection kit essentially as previously described (17,49,50). Cells were transfected with plasmid DNA prepared using an EndoFree plasmid kit (Qiagen). DRG neurones were obtained from E15 rat embryos and grown on poly-D-lysine- and laminin - coated coverslips in Neurobasal media and B27 supplement containing 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 0.1 μg/ml nerve growth factor (Sigma). Cells were transfected using Lipofectamine 2000. Briefly, media from 7-day-old neurones grown in 12-well dishes was removed and replaced with 0.5 ml Optimem (Invitrogen) containing 2 μg plasmid DNA and 3.5 μl Lipofectamine 2000. Cells were incubated for 4 h, and the Optimem was then removed and replaced with the conditioned Neurobasal/B27 medium.

Cells were fixed and processed for immunofluorescence microscopy 40 h post transfection. Briefly, cells were fixed in 4% (w/v) paraformaldehyde in PBS for 20 min, permeabilized in 0.1% (w/v) Triton X-100 in PBS for 10 min, blocked with 5% (v/v) FBS/0.1% (w/v) Tween-20 in PBS for 1 h, and then probed with primary antibodies diluted in blocking solution.

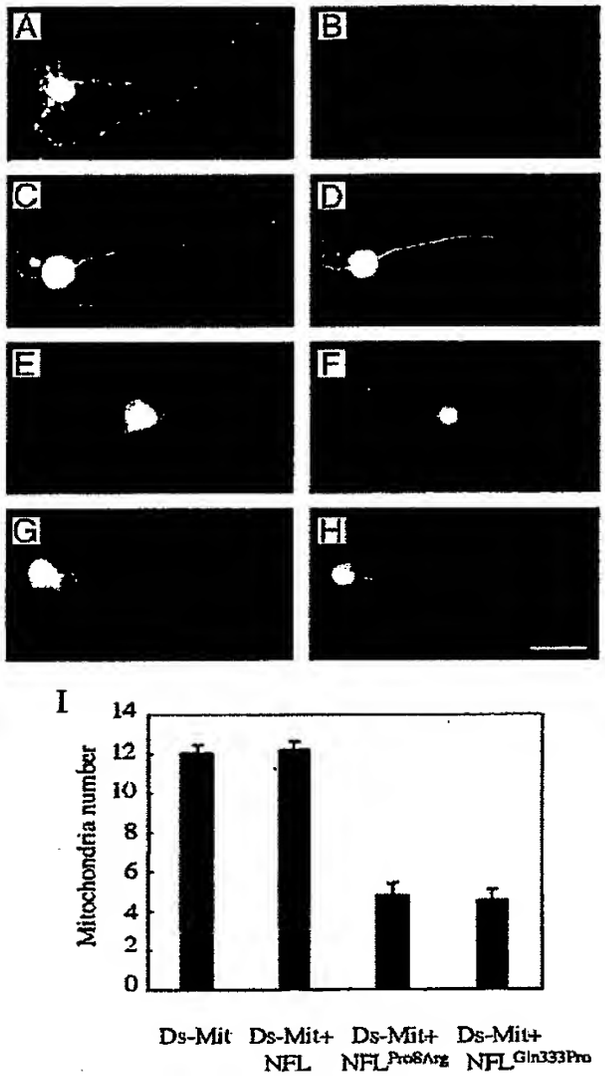


Figure 6. Aberrant localization of mitochondria in DRG neurones expressing NF-L^{Pro8Arg} and NF-L^{Gln333Pro}. Cells were transfected with DsRed2–Mito (Ds-Mit) either alone (A and B) or with human clones for wild-type NF-L (C and D), NF-L^{Pro8Arg} (E and F) or NF-L^{Gln333Pro} (G and H). (A), (C), (E) and (G) show DsRed2–Mito fluorescence; (B), (D), (F) and (H) show human NF-L. Scale bar = 40 μm. (I) Quantification of mitochondria distribution in a segment of axon 50 μm in length beginning 50 μm from the cell body. Expression of wild-type NF-L has no effect on the distribution of mitochondria, but expression of either NF-L^{Pro8Arg} or NF-L^{Gln333Pro} significantly (one-way ANOVA $P < 0.001$) reduces mitochondria numbers in this segment. Error bars are SEM.

NF-L was detected using antibody NA1214 (Affiniti) and human-specific monoclonal antibody anti-NF70 (Chemicon). NF-M/NF-H were detected using antibody SMI32 (Sternberger Monoclonals Inc.) and NF-M using rabbit antibody NF-M-Cterm (Chemicon). Primary antibodies were then detected using goat anti-mouse or goat anti-rabbit immunoglobulins coupled to Oregon Green or Texas Red (Molecular Probes) and the samples were mounted in Vectashield (Vector Labs). Cells were examined using a Zeiss Axioskop microscope and images were collected via a CCD camera (Princeton Instruments).

Analyses of bulk neurofilament transport in rat cortical neurones

Bulk transport of neurofilaments was analysed using a previously described assay (17). Briefly, neurones cultured for 7 days were co-transfected with EGFP–NF-M and human wild-type NF-L, NF-L^{Pro8Arg} or NF-L^{Gln333Pro}, and the cells were then fixed in 4% paraformaldehyde as described above 140, 200, 240 and 260 minutes post transfection. Cells were immunostained for human NF-L using antibody anti-NF70 in order to confirm co-expression, and images of EGFP–NF-M transfected cells were then collected (≥30 images per time point) via a CCD camera. Measurements of the distance travelled by EGFP–NF-M were calculated using Metamorph image analysis software. In these studies, measurements of the distance travelled by EGFP–NF-M were taken from the cell body to the front of the fluorescent signal and were of the longest distances in each neurone. The fluorescent front was taken as the most distal point at which fluorescence above background was detected. For neurites that exhibited branching, measurements were of the major neurite as determined by length and brightness of fluorescence. Since we measured the distance travelled by EGFP–NF-M for <300 μm from the cell body, and since the average length of the major neurites in the transfected cortical neurones >700 μm, this assay of neurofilament movement is of transport within neurites and is not a reflection of EGFP–NF-M in neurite terminals and neurite growth rates [for further discussion on this point see (17)]. To simplify presentation of the data and to facilitate comparisons between different experiments, the distance travelled by EGFP–NF-M at the first (140 min) time point is adjusted to zero. Statistical analyses of neurofilament transport were performed using one-way ANOVA tests.

Quantification of mitochondria in rat DRG neurones

For analyses of mitochondria distribution, DRG neurones were transfected with the mitochondrial marker DsRed2–Mito either alone or in co-transfections with human wild-type NF-L, NF-L^{Pro8Arg} or NF-L^{Gln333Pro}, and were fixed for analyses 40 h later. Following immunostaining for human NF-L with anti-NF-70 and Oregon Green-conjugated anti-mouse immunoglobulins, mitochondria were visualized using the *Discosoma* red fluorescent protein. Mitochondrial distribution was analysed using a modification of the method previously described for such analyses in N2a cells (27). Briefly, images of transfected cells (≥30 cells per transfection) were captured, and a defined area of axon, 50 μm in length beginning 50 μm from the cell body was circumscribed manually using Metamorph. The number of mitochondria in this segment of axon were then visualized and counted using the threshold function of Metamorph. Statistical analyses of mitochondria distribution were performed using one-way ANOVA tests.

Neurofilament preparation, SDS–PAGE and immunoblotting

Triton X-100-insoluble and -soluble fractions were prepared from SW13− cells transfected with NF-L, NF-M and NF-H. To do so, cells were scraped into PBS containing 0.5% Triton X-100, 0.6 M KCl, 5 mM EDTA, 5 mM EGTA, 40 µg/ml leupeptin (Sigma) and Complete protease inhibitor cocktail (Roche). The cells were then homogenized using a motorized homogeniser and the sample was spun at either 15 000*g* (average) for 15 min or 100 000*g* (average) for 30 min. Supernatants and pellets were separated and prepared for SDS–PAGE by addition of SDS–PAGE sample buffer and heating in a boiling water bath. Equal proportions of these fractions, each representing the same number of cells, were analysed by SDS–PAGE and immunoblotting. NF-L, NF-M and NF-H were detected using antibodies NA1214, NA1216 and NA1211 (Affiniti) respectively. The amyloid precursor protein (APP), a membrane-associated protein that is soluble in Triton X-100, was detected using antibody 22C11 (Roche Molecular Biochemicals), and was used as a control.

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See the related article beginning on page 507.

Oxidative stress and diabetic neuropathy: a new understanding of an old problem

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Diabetes has reached epidemic proportions in the Western world. In the United States, 17 million individuals have diabetes, greater than 6% of the population (1). The morbidity and mortality of diabetes is due to the development of both macrovascular and microvascular complications (2). Macrovascular complications including myocardial infarction, stroke, and large vessel peripheral vascular disease are 2 to 4 times more prevalent in individuals with diabetes. The underlying common factor in macrovascular complications is the ability of the diabetic condition to accelerate atherogenesis. Atherogenesis is a multifactorial response of vessels to injury; both insulin resistance and elevated lipid levels, common in diabetes, are primary triggers of atherogenic injury (3). The endothelium in diabetic arteries is also more prone to atherogenic injury, likely due to decreased production of endothelial nitric oxide, known to be antiatherogenic, and increased production of plasminogen activator inhibitor-1 (PAI-1) (4). While macro-vascular complications are common among diabetics, diabetes-specific microvascular complications will eventually affect nearly all individuals with diabetes. Diabetic retinopathy is the most common cause of adult blindness in the United States. Ninety percent of diabetics present evidence of retinopathy within 15 years of disease onset and approximately 25,000 new cases of diabetes-related blindness are reported per year (5). Diabetes is also the leading cause of renal failure in the United States, accounting for 40% of new cases each year (6). Greater than half of all patients with diabetes develop neuropathy, a progressive deterioration of nerves resulting in peripheral and autonomic nerve dysfunction. As a result, diabetic neuropathy is the most common cause of nontraumatic amputations and autonomic failure (7, 8). In his or her lifetime, a diabetic patient with neuropathy has a 15% chance of undergoing one or more amputations (9).

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Nonstandard abbreviations used: plasminogen activator inhibitor-1 (PAI-1); advanced glycation end-product (AGE); nerve growth factor (NGF); neurotrophin-3 (NT-3).

What are the mechanisms that underlie the development of microvascular complications?

Similar to our understanding of macrovascular complications, it is becoming increasingly clear that microvascular complications share a common pathophysiology. Animal and in vitro experiments over the last 25 years have implicated four major pathways of glucose metabolism in the development of microvascular complications (10). These include: 1) increased polyol pathway activity leading to sorbitol and fructose accumulation, NAD(P)H-redox imbalances, and changes in signal transduction; 2) nonenzymatic glycation of proteins yielding advanced glycation end-products (AGEs); 3) activation of PKC thereby initiating a cascade of stress responses, and 4) increased hexosamine pathway flux (1, 2, 10, 11). While specific inhibitors of each pathway block one or more diabetic microvascular complications, only recently has a link been established that provides a unified mechanism of tissue damage. Each pathway becomes perturbed as a direct or indirect consequence of hyperglycemia-mediated superoxide overproduction by the mitochondrial electron transport chain. Either inhibition of superoxide accumulation or euglycemia restores the metabolic and vascular imbalance and blocks both the initiation and progression of complications (2, 10, 12).

In the diabetic state, unchecked superoxide accumulation and resultant increases in polyol pathway activity, AGE accumulation, PKC activity, and hexosamine flux trigger a feed-forward system of progressive cellular dysfunction (Figure 1). In nerve, this confluence of metabolic and vascular disturbances leads to impaired neural function and loss of neurotrophic support, and long term, can mediate apoptosis of neurons and Schwann cells, the glial cells of the peripheral nervous system (13–15). Decreases in nerve growth factor (NGF), neurotrophin-3 (NT-3), ciliary neurotrophic factor, and IGF-I in nerves from animals with experimental diabetes are well documented and correlate with the presence of neuropathy (16–18).

Hedgehog proteins and diabetic neuropathy

The elegant work of Calcutt and colleagues in this issue of the *JCI* reports a decrease in desert hedgehog expression in nerves from young adult rats with streptozotocin-induced diabetes (19). Hedgehog proteins (sonic, desert, and indian) are essential for normal nervous system development (20). Desert hedgehog is found exclusively in the peripheral nervous system in Schwann cells and is important in peripheral nerve patterning (20). After 10 weeks of experimental diabetes, Calcutt et al. observed a decrease in desert hedgehog gene expression. This decrease correlates with several well established physiological and biochemical markers of experimental diabetes, including slowed motor and sensory nerve conduction velocities, decreased nerve blood flow, decreased pain threshold

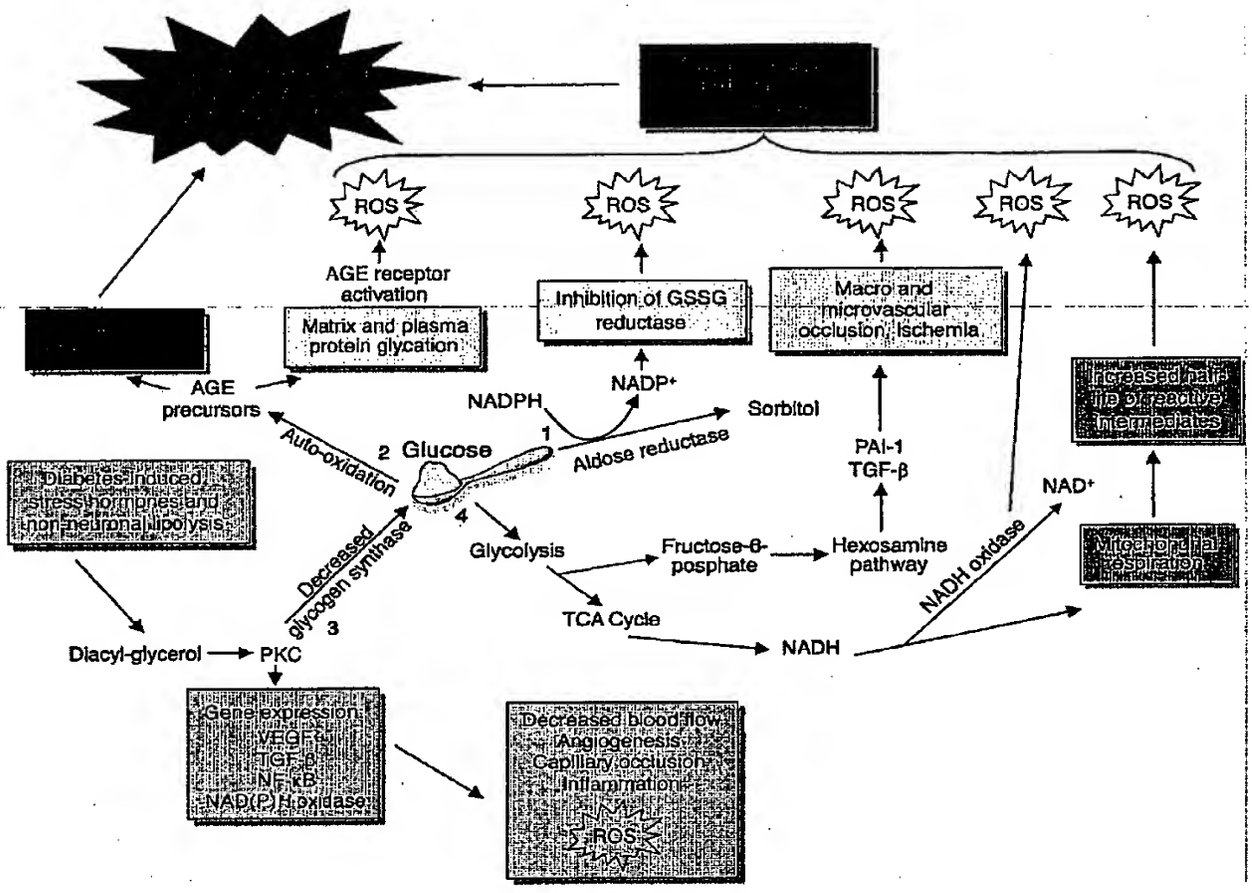

Figure 1

Mechanisms leading to neuronal degeneration in hyperglycemia involve reactive oxygen species (ROS) formation. The diabetic state produces impaired neurotropism, axonal transport and gene expression through at least four major pathways. 1) Excess glucose is diverted away from glycolysis by the polyol pathway that depletes NADPH and cellular antioxidant capacity. 2) Glucose also may become oxidized and form AGEs that alter extracellular matrix, activate receptors that produce ROS intermediates, and alter intracellular protein function. 3) PKC becomes activated either directly by glycolytic intermediates or indirectly as shown as a second messenger for stress hormones, leading to increased vascular disease, inflammation, and oxidative stress. 4) Partial glycolysis causes accumulation of glycolytic intermediates and leads to escape of fructose-6-phosphate along the hexosamine pathway that increases vascular disease and further ROS generation. These mechanisms are ultimately linked to superoxide production through increased glucose respiration that produces superoxide in the mitochondria and also activates the superoxide-producing NADH oxidase. GSSG, glutathione disulfide; TCA, tricarboxylic acid cycle.

in response to heat and/or formalin, and decreased NGF and neuropeptide levels. Thrice weekly injections of sonic hedgehog linked to an IgG fusion protein, beginning after 5 weeks of experimental diabetes and continuing for an additional 5 weeks, restored motor and sensory nerve conduction velocities and both NGF and neuropeptide levels. There was no therapeutic effect on nerve blood flow or pain threshold levels. Morphometric analyses of sciatic nerves revealed that diabetic animals had a decrease in medium sized myelinated fibers, which was restored by sonic hedgehog treatment.

Combination therapy for the treatment of diabetic neuropathy

While purely speculative, it is likely that restoration of hedgehog activity provided much needed neurotrophic support both directly, by activating hedgehog downstream pathways and indirectly, by restoring NGF levels. As discussed above, hyperglycemia-induced decreases in neurotrophic factors are well documented, with neurotrophic replacement frequently restoring one or more impaired nerve parameters to normal. Administration of NGF restores neuropeptide levels and sensory amplitudes in experimental diabetes (17, 21); in parallel, NT-3 normalizes nerve conduction slowing (22, 23) and IGF-I administration blocks the development of neuropathy and reverses impaired nerve regeneration (24, 25). When oxidative stress is induced in nerves of nondiabetic animals by administering pro-oxidants, decreases in NGF and NT-3 are observed similar to those reported in animals with experimental diabetes (26). Antioxidant therapy in experimental diabetic neuropathy blocks the observed decreases in nerve NGF and restores nerve function (27). Antioxidant therapy also restores normal blood flow and nerve conduction velocities in experimental diabetes

(11, 12, 28–32). Interestingly, neurotrophic factors may also serve as antioxidants and this function may contribute to their role as possible therapeutic entities in diabetic neuropathy (33–36).

Currently, there are no treatments for neuropathy other than treating the diabetic condition per se. Our improved understanding of the pathogenesis of diabetic neuropathy should assist in the development of new therapies (7, 8, 10). Clearly, therapeutic efficacy in man will be more challenging than in animal models of experimental diabetes. Damage to the nervous system by diabetes in man is more chronic, extensive, and severe (7, 8, 10). Several clinical trials have already failed to show improvement of diabetic neuropathy in patients with type 1 and 2 diabetes (reviewed in ref. 1). For example, there is no therapeutic benefit of acetyl-carnitine, aldose reductase inhibitors, or NGF in human diabetic neuropathy (1). What is required is combination therapy. Similar to how the oncologist approaches cancer, the endocrinologist and/or neurologist could, in the future, approach diabetic neuropathy. By blocking multiple pathway components (Figure 1), multiple causes of oxidative stress would in turn be blocked, preventing nervous system injury. If coupled with additional antioxidant therapy or neurotrophic support, this type of "complication cocktail" could provide the first effective treatment for diabetic neuropathy.

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Editorials

Diabetes and dementia

Is the brain another site of end-organ damage?

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Diabetes mellitus (DM) is an appalling disorder—quiet enough at onset, but with a toll on the body that is immense, causing damage to eyes, limbs, kidneys, and heart. Now Ott et al., in this issue of *Neurology*, provide compelling evidence that the brain too is damaged and that type 2 DM increases risk of dementia.[1] Such has been suspected for some time. Case–control and prospective studies have suggested that cognition is impaired in patients with diabetes,[2] and cross-sectional studies, including the earlier report from the Rotterdam group,[3] have suggested that diabetes is a risk factor for dementia. What was needed was large, prospective, population-based studies examining diabetes as a risk factor for dementia. The study now reported fulfills these demanding epidemiologic criteria amply, following a population of over 6,000 for up to 6 years, and demonstrating that the risk of dementia is nearly doubled in those with diabetes.

If, as seems to be the case, diabetes is responsible for causing dementia, then what is the mechanism? One obvious explanation would be that it is not the diabetes per se but the vascular complications of diabetes that result in neurodegeneration. Some earlier studies have found that diabetes increases the

risk of vascular dementia, although studies such as those tended to pass over the fact that mixed vascular and AD pathology is common and vascular dementia in the absence of AD changes is rare.[4] The Rotterdam study is unable to fully address whether the dementia associated with diabetes was vascular in origin; although only a minority had vascular dementia according to well-recognized criteria, neuroimaging was performed in only a small proportion. In any case, the distinction may not be important as it has become increasingly clear that vascular risk factors increase the risk of AD itself.[5] Ultimately, it is only with neuropathologic studies that the influence of risk factors on specific pathology can be answered, with one recent such study suggesting that diabetes does not increase the plaques and tangles of AD.[6]

Despite this, there are indications that the effect of diabetes on dementia is not, or at least not entirely, mediated through vascular factors. In the Rotterdam study, careful evaluation of cardiovascular risks was performed; these factors, although known to increase risk of dementia, did not account for a significant proportion of the risk attributable to the diabetes. In other studies, adjusting for evidence of clinical vascular disease did not obliterate the effect of diabetes on cognitive impairment.[2] In one prospective study where a hypertension–diabetes interaction was observed, this was partial—whereas some of the effects of diabetes on cognition could be attributable to coexisting hypertension, there was some evidence of memory impairment in the diabetic group independent of vascular factors.[7]

If not by vascular factors, then how does diabetes increase risk? As in much basic AD research, the problem is not one of finding potential mechanisms but of eliminating them. The demonstration that insulin resistance is associated with risk[8] is important as it might suggest that it is not the long term sequelae of diabetes, such as cardiovascular abnormalities, that results in neurodegeneration, but something to do with the action of insulin or the regulation of glucose metabolism. Perhaps the mechanism is mediated through the advanced glycation end products associated with diabetes and found in AD brain in both plaques and tangles. Alternatively, the mechanism might be mediated through insulin signaling. Neuronal insulin receptor resistance and subsequent cerebral glucose metabolism abnormalities have been suggested,[9] leading to the proposal that AD is "brain-type diabetes."[10]

However, insulin signaling regulates more than glucose metabolism. In AD, neurofibrillary tangle formation correlates with cognitive impairment and is associated with the loss of microtubules. Tangles form when the microtubule-associated protein tau self-aggregates in a highly phosphorylated form—a form that fails to bind and hence stabilize microtubules. Neuropathology suggests that the deposition of highly phosphorylated tau is one of the earliest signs of AD and cellular studies have demonstrated that in neurons one particular enzyme is predominantly responsible for this phosphorylation—glycogen synthase kinase-3ß (GSK-3ß).[11] Returning in a satisfyingly complete circle to diabetes, GSK-3ß was discovered as a downstream target of insulin signaling; insulin stimulates the PI3 kinase/protein kinase B (PKB) pathway, which in turn inhibits GSK-3ß. It follows that effective insulin signaling would be expected to reduce tau phosphorylation and increase the stability of microtubules, and in cultured neurons, this is exactly what is found.[12] Yet more exciting evidence that insulin signaling through PKB is important in AD came from the recent demonstration of an interaction between the presenilin (PS) proteins and PKB. Mutations in PS-1 causing AD decreased PKB activity leading to an increase in GSK-3ß activity.[13]

That a failure of insulin signaling and mutations in PS-1 both result in decreased inhibition of the tau-kinase GSK-3ß is intriguing. An important question raised by these studies regards the effects of insulin signaling on amyloid precursor protein metabolism, and further work will be needed to

determine the place of insulin signaling in the cascade of events between amyloid formation and tau aggregation. Other questions relate to the interaction between diabetes and genetic risk factors. No doubt the Rotterdam group will examine the interaction between *APOE* and diabetes in influencing risk, but other gene/internal–environment interactions could be important. Another putative genetic risk factor for AD is the gene encoding angiotensin converting enzyme (ACE)[14] and diabetologists have already noted that polymorphic variation in ACE predisposes to insulin resistance and influences end-organ response to diabetes.[15,16] Might ACE be a common factor between AD and diabetes?

Whether the epidemiology is linked with the molecular studies or not, one obvious conclusion from this important study might have been that a new therapeutic approach had been found. It would be marvelous if we were able to proclaim that treating diabetes could be added to reducing blood pressure as a potential preventative strategy. Unfortunately, Ott et al. find the highest risk of dementia in those receiving insulin. Being treated with insulin might simply be a surrogate marker for diabetic severity, but it does suggest that we will have to understand more of the mechanisms of this important association before the public health campaigns get going.

Footnotes

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Invited article

Cerebral complications of diabetes: clinical findings and pathogenetic mechanisms

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Abstract

This review describes the cerebral complications of diabetes mellitus from a neuropsychological, neurophysiological and neuroradiological perspective. In addition, possible pathogenetic mechanisms are discussed. Neuropsychological studies of diabetic patients generally report modest deficits in learning and memory and information processing. Notably, in elderly diabetic patients cognitive deficits may be more prominent. Recent epidemiological studies show that in the elderly diabetes is associated with an increased risk for dementia. Neurophysiological studies show increased latencies of evoked potentials and event-related potentials. Neuroradiological findings are enhanced peripheral and central cerebral atrophy, as well as focal lesions.

The pathophysiology of the effects of diabetes on the brain has not been fully elucidated. The putative involvement of cerebral metabolic and microvascular disturbances, similar to those implicated in the pathogenesis of peripheral diabetic neuropathy, is discussed. In addition, the role of repeated hypoglycaemic episodes, cerebrovascular disease and hypertension is addressed. Finally, the potential differential effects of insulin dependent and non-insulin dependent diabetes on the brain are discussed, as well as possible links with brain ageing.

Keywords: Diabetes mellitus; Brain; Cognitive function; Ageing; Evoked potentials; Magnetic resonance imaging

Peripheral neuropathy is a well-known complication of diabetes mellitus [1,2]. In recent years evidence is emerging that diabetes also affects the central nervous system [3–5]. Both acute and chronic metabolic and vascular disturbances can impair the functional and structural integrity of the brain in diabetic patients. For example, diabetes increases the risk for stroke [6,7] and stroke outcome is worse in diabetic patients [8]. In addition, hyper- and hypoglycaemic episodes may result in acute cerebral dysfunction [9–11]. The consequences of these acute insults to the brain are well recognised and have been reviewed previously [8,11,12]. The present paper will focus on functional and structural cerebral alterations that develop more insidiously and tend to be more subtle. Recent neuropsychological, neurophysiological, and neuroradiological studies into the nature and the magnitude of the long-term

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effects of diabetes on the brain will be reviewed and possible pathogenetic mechanisms will be discussed.

Cerebral manifestations of diabetes

Cognitive function

Neuropsychological studies in diabetic patients have reported variable performance deficits on a wide range of cognitive tests. Problems with learning and memory, problem solving and mental and motor speed have been noted [13–21]. The nature and severity of cognitive deficits in diabetic patients appears to be dependent on age and possibly also on the type of diabetes (insulin dependent diabetes (IDDM) versus non-insulin dependent diabetes mellitus (NIDDM)).

Cognitive function in children with IDDM

In diabetic children age at diabetes onset and hypoglycaemic episodes appear to be the prime determinants of cognitive changes; children and adolescents who develop diabetes before 5 years of age [22,23] and children who frequently experience hypoglycaemic episodes [23–25] are more likely to express cognitive deficits. Visual and spatial abilities may even be affected by asymptomatic recurrent hypoglycaemia [24]. Disease duration and poor glycaemic control, as determined by HbA1 values, may be additional factors influencing cognitive function in diabetic children [22,26].

Cognitive function in adult IDDM patients

In adult IDDM patients small but detectable reductions in mental efficiency have been reported repeatedly [22]. Severe deficits occur only in few patients [27]. The pattern of impairment across different cognitive tasks tends to vary among studies [18]. This variation might be due to the relatively subtle nature of the cognitive deficits, as well as the heterogeneity of study populations. In addition, exposures to different risk factors such as frequent hypoglycaemic episodes, on the one hand, and chronic hyperglycaemia, on the other, may vary between patients.

Glycaemic control plays a dual role in the prevention of cerebral complications in adult IDDM patients. On the one hand poor glycaemic control, as reflected in increased HbA1 values, is associated with cognitive dysfunction [22,28]. Moreover, the development of other complications associated with poor glycaemic control, such as peripheral neuropathy, is associated with cognitive dysfunction [16]. On the other hand, intensive treatment increases the frequency of hypoglycaemic episodes [29,30], and may thus adversely affect the brain. In adult IDDM patients, the frequency of severe hypoglycaemic episodes has been shown to be inversely correlated with performance in cognitive tests [19,22,31–34]. It should be noted, however, that such a correlation has not been demonstrated invariably [17,29,35] and that the relation between repeated hypoglycaemic episodes and cognitive dysfunction remains subject to ongoing debate [36].

Cognitive function in NIDDM patients

Compared to IDDM, neuropsychological studies in NIDDM patients have provided more consistent results (Review [20,21]). In NIDDM patients moderate degrees of cognitive impairment have been reported, particularly in tasks involving verbal memory or complex information processing. Tasks that tap basic attentional processes, motor reaction time and immediate memory appear to be unaffected [20,21]. Although the exact impact on daily functioning remains unclear, the fact that differences between NIDDM patients and age-matched controls can be detected with relatively crude tests such as the mini mental state examination [37–40], suggests that the deficits are not insignificant.

Risk factors for cognitive dysfunction in NIDDM are increased HbA1 and fasting plasma glucose levels [41,42], elevated serum triglyceride levels [43], and the presence of peripheral neuropathy [41]. Since severe hypoglycaemic episodes are relatively uncommon in NIDDM patients [44,45], they do not appear to be a prime determinant of cognitive dysfunction in NIDDM.

Diabetes, hyperinsulinaemia and impaired glucose tolerance in the elderly

The prevailing view of the studies that have been discussed thus far is that cognitive functions can be impaired in diabetic patients, in particular those with NIDDM, but that the impact of these impairments on

day-to-day functioning appears to be limited. Although this conclusion may be valid for relatively young (age < 70 years) patients, it does not appear to hold true for the elderly. Recent epidemiological studies in elderly subjects have demonstrated an association between diabetes and dementia [46,47]. This association was evident for Alzheimer's disease (relative risk in diabetic patients ~1.5 [46,47]) as well as for vascular dementia (relative risk in diabetic patients ~2 [46]). These findings are in line with previous studies in elderly diabetic patients which show relatively marked deficits in cognitive functions compared to age matched controls [38–40,48–50]. The reasons why the effects of diabetes on the brain appear to be more prominent in the elderly are unclear. Importantly, cognitive functions in the elderly may be impaired in subjects with newly diagnosed diabetes [38], as well as in subjects with impaired glucose tolerance [38,51] and/or hyperinsulinaemia [38,51–53]. These observations indicate that the effects of diabetes on the brain in the elderly may be related to an increased vulnerability of the ageing brain to the diabetic condition rather than to a prolonged exposure to diabetes. Although at present there is insufficient evidence to support a causal relationship between alterations in glucose metabolism and cognitive dysfunction in the elderly, recent studies implicating impaired glucose handling in the pathophysiology of Alzheimer's disease [54,55] stress the necessity of further investigations into the relation between glucose, insulin and the brain.

The question arises whether cognitive impairments in diabetic patients may reflect a central equivalent of peripheral diabetic neuropathy. The next section of this review will describe neurophysiological and neuroradiological evidence for the existence of such a 'central neuropathy', or encephalopathy. It should be considered, however, that in addition to such an encephalopathy other factors might influence cognitive function in diabetic patients. For example, the prevalence of psychiatric disorders, in particular depressive and anxiety disorders, is increased in both IDDM and NIDDM [56,57]. This increased prevalence of depression in diabetes can result from an inability to cope with the stresses associated with diabetes, but alterations in monoaminergic function in the brain that are known to be associated with diabetes could also be involved [57–59].

Electrophysiological abnormalities

Evoked potentials

Evoked potentials are the electrophysiological manifestations of the brain's response to an external stimulus, such as a flash of light or a sound click. Measurement of the latency of evoked potentials of different modalities, including visual evoked potentials (VEPs), brainstem auditory evoked potentials (BAEPs), and somatosensory evoked potentials (SSEPs), have been widely used to examine the functional integrity of the central nervous system in diabetic patients [60].

In the BAEP five waves can be distinguished, designated wave I through V. Wave I, III and V are considered to reflect activity in the acoustic nerve, the pons and the midbrain, respectively [61]. In both IDDM and NIDDM patients the latency of wave I [62,63], as well as the interpeak latencies I–III and III–V, were increased [63–66]. The latency of the VEP P100 wave, which is thought to be generated in the visual cortex [67], was increased both in IDDM and in NIDDM patients [62,68–70]. P100 latencies correlated positively with the duration of diabetes and HbA1 levels [71,72] and could be improved by intensive insulin treatment [70].

Studies on SSEPs in diabetic patients have provided more variable results. Increased latencies of the central components of the SSEP have been reported [73], although most studies have only found significant conduction delays in peripheral components of the somatosensory pathways [66,74,75].

Event-related potentials

In addition to evoked potentials, the latencies of event-related potentials, such as the P300 wave are increased in both IDDM and NIDDM patients [13,76–78]. The P300 wave is a late cortical neurophysiological event associated with cognitive and mnemonic functions [79,80]. It is considered to reflect neuronal events underlying information processing and is strongly associated with attention and short-term memory [79,80]. The increased P300 latency in diabetic patients may reflect impairment of

higher brain functions, thus providing a link between electrophysiological and cognitive impairments.

Neuroradiological changes

Several studies have identified diabetes as a risk factor for the development of cerebral atrophy and focal white matter lesions (e.g. [81–83]). These observations have been substantiated in studies that specifically compared IDDM and NIDDM patients to age-matched controls. Cerebral atrophy, diagnosed on the basis of widened sulci and/or enlarged lateral ventricles, was more prevalent in diabetic patients [84–86]. In addition, focal lesions were observed in 69% of a group of IDDM patients, whereas comparable lesions were observed in only 12% of an age-matched control group [83]. It has been suggested that the radiological appearance of the brain in diabetic subjects mimics that of normal ageing, but appears to develop at a younger age than in non-diabetic subjects [85].

Pathogenetic mechanisms

The aforementioned studies demonstrate that diabetes can impair cerebral function and structure. Several pathogenetic mechanisms could be involved. Firstly, factors that are also implicated in the pathogenesis of peripheral diabetic neuropathy could play a role. Secondly, as the neuropsychological and neuroradiological alterations in diabetes mimic those observed in the ageing brain [21,85], and as elderly individuals appear to be more susceptible to the effects of diabetes on the brain than younger subjects, it may be suggested that diabetes interacts with, or accelerates, the ageing process of the brain. Thirdly, risk factors for cerebrovascular events such as hypertension and atherosclerosis may be involved [87–89]. Finally, repeated hypoglycaemic episodes could play a role, in particular in IDDM [22,36].

The next part of this review focuses the aforementioned pathogenetic mechanisms. Studies into the pathogenesis are mostly based on animal models, in particular the streptozotocin (STZ)-induced diabetic rat and the BB/Wor rat. STZ destroys pancreatic β-cells relatively selectively, leading to insulin deficiency and hyperglycaemia [90]. In the BB/Wor rat diabetes develops spontaneously, secondary to an immune-mediated destruction of the β-cells [91]. These models of IDDM have been used extensively to examine the pathogenesis of peripheral neuropathy (Review [92]), but can also be used to study the effects of diabetes on the brain [93,94]. Thus far, few studies have employed animal models of NIDDM.

Links with the pathogenesis of diabetic neuropathy

The pathogenesis of peripheral diabetic neuropathy is multifactorial, involving metabolic changes [95], neurovascular dysfunction [96] and changes in trophic support [97]. Metabolic changes include an increased flux of glucose through the polyol pathway, leading to accumulation of sorbitol and fructose and depletion of myo-inositol [95]. Other metabolic changes are an enhanced non-enzymatic glycation of neural proteins [98] and an imbalance in the generation and scavenging of reactive oxygen species [99]. Vascular changes include reductions in nerve blood flow [100,101] leading to a decreased endoneurial oxygen tension [102]. Metabolic and vascular changes may be linked to reductions in peripheral nerve conduction velocity through reductions in Na^+/K^+-ATPase activity, leading to alterations in transmembrane ion gradients [103,104].

Like in the peripheral nervous system, increased glucose levels in the brain [105–107] lead to enhanced polyol pathway flux [108] and accumulation of sorbitol and fructose [109,110]. However, sorbitol and fructose levels appear to be lower than in peripheral nerves [109,110]. Remarkably, brain myo-inositol content is increased in diabetic rats [110], contrasting the myo-inositol decrease in peripheral nerves [95]. Enhanced non-enzymatic glycation of neuronal and non-neuronal proteins has also been demonstrated in the brain and spinal cord of diabetic rats [98,111,112], although the levels of glycation products in the central nervous system appear to be much lower than in peripheral nerves [98,113]. Also, increased concentrations of lipid peroxidation by-products, indicative of oxidative damage, have been demonstrated in the cerebral microvasculature and in brain tissue of diabetic rats [114–116]. Furthermore, the activity of superoxide dismutase and catalase, enzymes involved in the antioxidant defence of the brain, is decreased [116,117].

Functional and structural alterations in the cerebral microvasculature of diabetic animals include thickening of capillary basement membranes, decreased capillary density [118–120] and regional decreases in cerebral blood flow [121–123]. In diabetic patients thickening of cerebral capillary basement membranes [124,125] as well as regional decreases [126,127] and increases [128,129] in cerebral blood flow have been reported.

Links with the pathogenesis of brain ageing

Several processes that have been implicated in the pathogenesis of diabetic complications are also implicated in brain ageing, including oxidative stress, non-enzymatic protein glycosylation and ischaemia [130,131].

Increased oxidation of proteins and lipids has been demonstrated in the brains of ageing rodents [132,133] and humans [134]. Ageing is also associated with an accumulation of advanced glycosylation end products (AGEs) in various tissues, possibly as a result of lower protein turnover [130]. Interestingly, the formation of AGEs is associated with the increased production of reactive oxygen species [130,135], thus linking non-enzymatic glycosylation to increases in oxidative stress. Finally, brain capillaries may undergo progressive degeneration during ageing, caused by amyloid deposits, thickened basement membrane, and reduced vessel elasticity [136,137]. In the long term, capillary abnormalities may lead to increased capillary resistance, which in turn can affect cerebral blood flow. The adverse effects of oxidative stress, AGEs and ischaemia may be partially mediated by disturbance of neuronal calcium homeostasis [131,138]. Sustained alterations in neuronal calcium homeostasis are suggested to present a final common pathway in the development of the neuropathological changes associated with brain ageing [139,140]. Like ageing, diabetes is associated with impairment of neuronal calcium homeostasis [131,141].

Obviously, the relative contribution of ischaemia, oxidative stress, the formation of AGEs and disturbance of neuronal calcium homeostasis in brain ageing and the development of cerebral complications of diabetes differs. However, the similarities are apparent and may explain part of the increased susceptibility of elderly diabetic patients to the effects of diabetes on the brain.

Cerebrovascular alterations

Diabetes is associated with an increased prevalence of hypertension [142,143] and cerebrovascular disease [12,144]. Cerebrovascular disease increases the risk of stroke [145] and may lead to haemodynamic alterations [12]. These haemodynamic alterations are reflected in the aforementioned regional alterations in cerebral blood flow. In addition, cerebral vasoreactivity is impaired in diabetic patients [146–148]. Cerebral vasoreactivity, and accompanying changes in blood flow, are important compensatory mechanisms during conditions such as hypoglycaemia, hypotension, hypoxia and hypercapnia. Loss of these compensatory mechanisms may have detrimental effects on the brain.

Hypertension may play both an indirect and a direct role in the pathophysiology of cerebral complications of diabetes. Hypertension accelerates the development of cerebrovascular disease [149]. In addition, hypertension predisposes to cognitive impairment in both non-diabetic [53,150] and diabetic [151] elderly. The pathophysiology of the effects of persistent hypertension on the brain is only partially understood. However, in the context of this review it is important to note that hyperinsulinaemia appears to potentate the adverse effects of hypertension on the brain, even in non-diabetic subjects [53].

Effects of hypoglycaemia

Cognitive changes that accompany a single episode of hypoglycaemia are considered to be transient [17]. However, repeated episodes of hypoglycaemia may lead to cumulative damage to the brain, causing permanent cognitive impairment [32,33]. Selective neuronal damage during hypoglycaemia has been shown to result from over-activation of a subtype of excitatory amino acid receptor, the *N*-methyl-D-aspartate(NMDA)-receptor, which is one of the main excitatory amino acid receptors on cerebral neurones [152]. NMDA-receptor over-activation leads to pathologically enhanced levels of free intracellular calcium [153,154], in turn leading to loss of nuclear and mitochondrial function and activation of pro-

teases and other calcium dependent enzymes [154–156].

Conclusions

Long-term diabetes mellitus can lead to cerebral disorders. Manifestations of these disorders include cognitive dysfunction, electrophysiological abnormalities and structural changes. The effect on daily functioning is generally assumed to be limited. However, in elderly diabetic patients impaired performance can be detected with tests like the mini mental state examination [37–40,48], suggesting that the deficits are not trivial. Moreover, epidemiological studies indicate that elderly diabetic patients are at increased risk for developing dementia [46,47]. The notion that the effects of diabetes on the brain are most marked in the elderly and mimic the effects of ageing provide a challenge for the clinician: the effects of diabetes need to be distinguished from those of 'normal ageing' to avoid underestimation of the impact of the effects of diabetes on the brain.

Thus far fewer studies have addressed the reversal of cognitive deficits in diabetic patients. Preliminary studies show that cognitive functions may improve with glycaemic control in elderly NIDDM patients [157–159]. It should be considered, however, that the balance between the potential harmful effects of chronic hyperglycaemia on the one hand and of repeated hypoglycaemic episodes on the other is delicate. In addition to optimising glycaemic control, increasing insight into the pathogenesis and identifying risk factors for cerebral dysfunction in diabetes may lead to the development of additional preventive and interventional measures. One of the key questions that needs to be answered is why the effects of diabetes on the brain appear to be more pronounced in the elderly. There are several possible explanations. Firstly, the aging brain may be more sensitive to the effects of diabetes due to a reduced 'cognitive reserve capacity' as a result of the wear and tear of ageing. In favour of this explanation is the finding that young adult IDDM patients express neurophysiological and neuroradiological deficits which are qualitatively similar to those in NIDDM patients, but do not appear to express clinically significant cognitive deficits. Alternatively, the pathogenetic processes of ageing and diabetes may interact, as was discussed in the previous section of this review, leading to an accelerated cognitive decline in the elderly. Finally, differences in the pathophysiology of IDDM and NIDDM may provide an explanation for the differential functional deficits in different age groups, as NIDDM is by far the most common form of diabetes in the elderly, whereas IDDM is the most common form in the younger population. For example, given the clustering of hyperinsulinaemia/insulin resistance with dyslipidaemia and hypertension in NIDDM [160,161], support for this latter hypothesis could be provided by the observation that hyperinsulinaemia potentiates the adverse effects of hypertension on the brain [53,151]. Moreover, even in relatively young NIDDM patients the pattern of cognitive deficits appears to be more consistent than in IDDM [76,162]. Still, as yet it remains uncertain whether ageing itself or differences in the pathophysiology of IDDM and NIDDM are the main determinant in the enhanced effects of diabetes on the brain in the elderly. The elucidation of the complex interplay between IDDM, NIDDM and ageing will provide a key challenge for future studies into the effects of diabetes on the brain.

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EDITORIAL

Insulin receptors and insulin actions in the nervous system

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Summary

Insulin receptors are widely distributed in the brain. They are also present in peripheral nerve. Insulin signaling through its receptors in the brain is responsible for the hormone's effects on the regulation of food intake, body weight, and reproduction. Signaling through the insulin receptor also appears to influence higher cognitive functions. In peripheral nerve, insulin signaling may play a role in the maintenance and repair of myelinated fibers. Future studies should determine the extent to which a defective insulin signal may be linked to the pathogenesis of diabetic neuropathies and neurodegenerative disorders such as Alzheimer's disease. Copyright © 2000 John Wiley & Sons, Ltd.

Keywords insulin receptors; insulin actions; central nervous system; peripheral nerve; diabetic neuropathy; Alzheimer's disease

The central nervous system

In 1978, Havrankova, Roth and Brownstein [1] demonstrated for the first time the presence of insulin receptors in the central nervous system (CNS), classically an 'insulin-insensitive' tissue. Since this seminal paper, numerous studies have been devoted to insulin receptors and insulin actions in the CNS. Insulin receptors are widely distributed in the brain, with much higher concentrations in neurons than in glia [for a review see 2]. They are found in both cell bodies and synapses. Distinct regional patterns of expression of the insulin receptor may reflect different functions.

One domain of insulin action in the CNS is related to the control of food intake through insulin receptors abundantly located in the olfactory bulb and thalamic nuclei. In fact, insulin was the first hormonal signal to be implicated in the control of body weight by the CNS [3]. Both insulin and leptin act as dual adiposity signals to the brain for the regulation of food intake and body weight [4]. Reduced CNS insulin delivery may be a feature of several different forms of obesity [5]. Like leptin, insulin signaling in the CNS plays a central role not only in participating in the regulation of food intake and body weight, but also in the regulation of reproductive function, as demonstrated very recently by Brüning *et al.* These researchers discovered that in addition to diet-sensitive obesity, mice with a neuron-specific disruption of the insulin receptor gene exhibit reduced fertility due to hypothalamic dysregulation of luteinizing hormone [6].

Another domain of insulin action in the CNS concerns cognitive functions. High levels of insulin receptors are present in the limbic system, particularly the hippocampus which is critically involved in spatial memory processing. Signaling through the insulin receptor appears to participate in this memory processing [7]. Acute intranasal administration of insulin has been shown to directly affect brain function [8]. In addition, defects in insulin action in the

CNS may be linked to the pathogenesis of neurodegenerative disorders such as Alzheimer's [9] and Parkinson's [10] disease. In a review article published in the preceding issue of *Diabetes/Metabolism Research and Reviews*, Ryan and Geckle discussed the roles of ageing, Type 2 diabetes and insulin in learning and memory dysfunction [11].

And the peripheral nervous system?

In 1987, Waldbillig and LeRoith [12] showed that peripheral sensory and autonomic ganglia contain insulin receptors. In the preceding issue of *Diabetes/Metabolism Research and Reviews*, Sugimoto *et al.* [13] demonstrated the presence of insulin receptors in the sciatic nerve and dorsal root ganglion. Using both light and ultrastructural immunochemistry, these researchers further localized insulin receptors to the nodal and paranodal axolemma and Schwann cell plasma membranes. These sites of myelinated fibers are known to possess specialized molecular structures such as ion channels, Na^+/K^+-ATPase, glucose transporters, aldose reductase, and specialized molecules such as paranodin and integrins. Thus, in peripheral nerve the insulin receptor co-localizes with membrane molecules believed to play important roles in the maintenance of nodal function and structure, fiber regeneration and repair of myelinated fibers. It is tempting to speculate that signaling through the insulin receptor is involved in these functions, as suggested by the neurotrophic properties of insulin and insulin-like growth factors.

What are the pathophysiological implications?

As pointed out by Sugimoto *et al.* [13], one of the key differences between the neuropathy in the two types of diabetes is the progressive disruption of the paranodal ion-channel barrier in Type 1 diabetes. This alteration has been associated with the more severe conduction defect in Type 1 compared to Type 2 diabetes. Decreased insulin signaling due to insulinopenia may play a role in the functional and structural abnormalities of the nodal and paranodal apparatus in Type 1 diabetes. Also, the co-localization of the insulin receptors and integrins in the Schwann cell suggests that a decreased insulin signal may impair functions of the integrins and contribute to Schwann cell/myelin alterations in diabetic neuropathy.

Two other findings in the article of Sugimoto *et al.* [13] are of particular interest. In endoneurial microvessels, immunoelectron microscopy showed insulin receptor localization on plasma membranes of endothelial cells and pericytes/vascular smooth muscle cells; and high intensity of immunostained insulin receptor was found in close proximity to interendothelial tight junctions. These findings are consistent with the localization of insulin receptor in brain capillaries where insulin crosses the blood–brain barrier via receptor-mediated transcytosis. Sugimoto *et al.* also suggest an additional pathway for insulin passage at the interendothelial tight junction, and the possibility that insulin signaling is involved in the integrity of the blood–nerve barrier.

Nothing is presently known about the regulation of the insulin receptor and insulin signaling in peripheral nerve. Previous studies [for a review see 2] have suggested that, unlike peripheral (i.e. adipocyte, liver and muscle) insulin receptors, the brain insulin receptors do not undergo downregulation after exposure to high concentrations of insulin. If this is also true for peripheral nerve, then one may anticipate that alterations in insulin receptor (and insulin receptor signaling) would not occur in peripheral nerve as a result of hyperinsulinemia *per se*. Downregulation of insulin receptors, however, may still occur at the level of the blood–nerve barrier, allowing lesser amounts of insulin to be delivered into the nerve. Some degree of insulin deficiency may therefore affect the peripheral nerve even in hyperinsulinemic situations.

Exactly how insulin action in the brain can influence higher cognitive functions such as learning and memory is not yet fully understood. In patients with Alzheimer's disease, insulin has been reported to be higher in plasma and lower in cerebrospinal fluid when compared to control subjects [14]. One possible explanation for this discrepancy may be a lower rate of CNS insulin delivery as a result of insulin receptor downregulation at the level of the blood–brain barrier, as discussed above. In addition to diminished insulin signal, insulin action may also be impaired as a result of reduced insulin receptor tyrosine kinase activity in the Alzheimer's disease brain [9]. Also, in cultured human neurons, insulin and IGF-1 have been shown to reduce the phosphorylation of tau [15], a neuronal microtubule-associated protein that, in its hyperphosphorylated form, is the major component of the neurofibrillary lesions in Alzheimer's disease.

Conclusion

Clearly, the nervous system, both central and peripheral, is an important target of insulin action in health and disease. Many studies make a strong case for direct effects of insulin on the nervous system through its signaling network. Delineating the extent to which impaired insulin signaling is directly implicated in the pathogenesis of diabetic neuropathies and of neurodegenerative disorders such as Alzheimer's disease, and elucidating the mechanisms involved, represent new and exciting avenues of investigation, and potential promises for future development of novel therapeutic approaches.

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Oxidative stress in Parkinson's disease and other neurodegenerative disorders.

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The cause of cell death in neurodegenerative diseases remains unknown but the formation of free radicals and the occurrence of oxidative stress may be a common component of many, if not all, such disorders. For example, in substantia nigra in Parkinson's diseases key alterations occur, in iron handling, mitochondrial function and antioxidant defences, particularly reduced glutathione. These indices of oxidative stress are accompanied by evidence of free radical mediated damage in the form of increased lipid peroxidation and oxidation of DNA bases. The alterations in oxidative stress occurring in Parkinson's disease appear not be related to the administration of L-DOPA. Some alterations of oxidative stress are found in other basal ganglia in degenerative disorders (multiple system atrophy, progressive supranuclear palsy, Huntington's disease) but these have not been investigated to the same extent. Similarly, examination of biochemical changes occurring in Alzheimer's disease, motor neurone disease and diabetic neuropathy also suggest the involvement of free radical mediated mechanisms as a component of neurodegeneration. It is probable that irrespective of the primary cause of individual neurodegenerative disorder, the onset of oxidative stress is a common mechanism by which neuronal death occurs and which contributes to disease progression. Clearly, therapeutic strategies aimed at limiting free radical production and oxidative stress and/or damage may slow the advance of neurodegenerative disease.

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